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SUPREME COURT, U.S.

No. 05-608

IN THE

Supreme Court of the United States

MEDIMMUNE, INC.,
Petitioner,

v.

GENENTECH, INC., *et al.*,
Respondents.

On Writ of Certiorari to the United States Court of Appeals for the Federal Circuit

JOINT APPENDIX
VOLUME II

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VOLUME II

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NOTICE

The following items have been omitted in printing this appendix because they appear in the following pages of the printed appendix to the petition for certiorari. They are incorporated herein by reference:

Amended memorandum of decision and order of the District Court (April 23, 2004) 21a

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Certificates of service whenever appended to documents appearing herein have been omitted in printing. Some royalty numbers have been redacted; such redactions are noted by ellipses.

United States Patent [19]

Boss et al.

[11] **Patent Number: 4,816,397**

[45] **Date of Patent: Mar. 28, 1989**

[54] **MULTICHAIN POLYPEPTIDES OR PROTEINS AND PROCESSES FOR THEIR PRODUCTION**

[75] Inventors: **Michael A. Boss**, Slough; **John H. Kenten**; **John S. Emtage**, both of High Wycombe; **Clive R. Wood**, Near Fordingbridge, all of United Kingdom

[73] Assignee: **Celltech, Limited**, Slough, United Kingdom

[21] Appl. No.: **672,265**

[22] PCT Filed: **Mar. 23, 1984**

[86] PCT No.: **PCT/GB84/00094**

§ 371 Date: **Nov. 14, 1984**

§ 102(e) Date: **Nov. 14, 1984**

[87] PCT Pub. No.: **WO84/03712**

PCT Pub. Date: **Sep. 27, 1984**

[30] **Foreign Application Priority Data**

Mar. 25, 1983 [GB] United Kingdom 8308235

[51] **Int. Cl.**[4] **C12P 21/00**; C12N 15/00; C12N 1/00; C12N 1/20

[52] **U.S. Cl.** **435/68**; 435/172.3; 435/243; 435/255; 435/320; 435/252.31; 435/252.33

[58] **Field of Search** 435/68, 172.3, 243, 435/320, 253, 255

[56] **References Cited**

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Haley et al., DNA, vol. 1, pp. 155-162, 1982.

Gough et al., Biochemistry, vol. 19, pp. 2702-2710, 1980.

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Seidman et al: "Immunoglobulin light-chain structural gene sequences cloned in a bacterial plasmid," *Nature, vol. 271, pp. 582-585, 1978.*

Primary Examiner—Blondel Hazel

Attorney, Agent, or Firm—Cushman, Darby & Cushman

[57] **ABSTRACT**

Multichain polypeptides or proteins and processes for their production in cells of host organisms which have been transformed by recombinant DNA techniques. According to a first aspect of the present invention, there is provided a process for producing a heterologous multichain polypeptide or protein in a single host cell, which comprises transforming the host cell with DNA sequences coding for each of the polypeptide chains and expressing said polypeptide chains in said transformed host cell. According to another aspect of the present invention there is provided as a product of recombinant DNA technology an Ig heavy or light chain or fragment thereof having an intact variable domain. The invention also provides a process for increasing the level of protein expression in a transformed host cell and vectors and transformed host cells for use in the processes.

18 Claims, 13 Drawing Sheets

U.S. Patent Mar. 28, 1989 Sheet 1 of 13 4,816,397

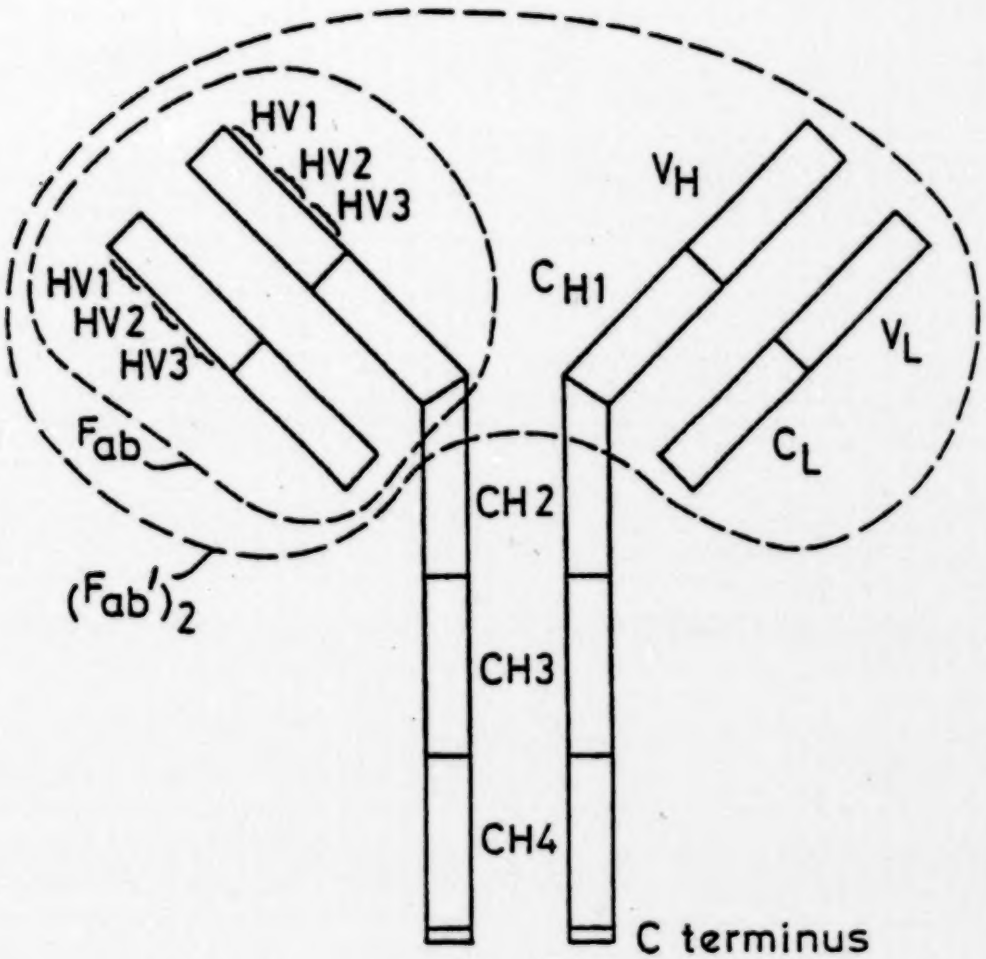
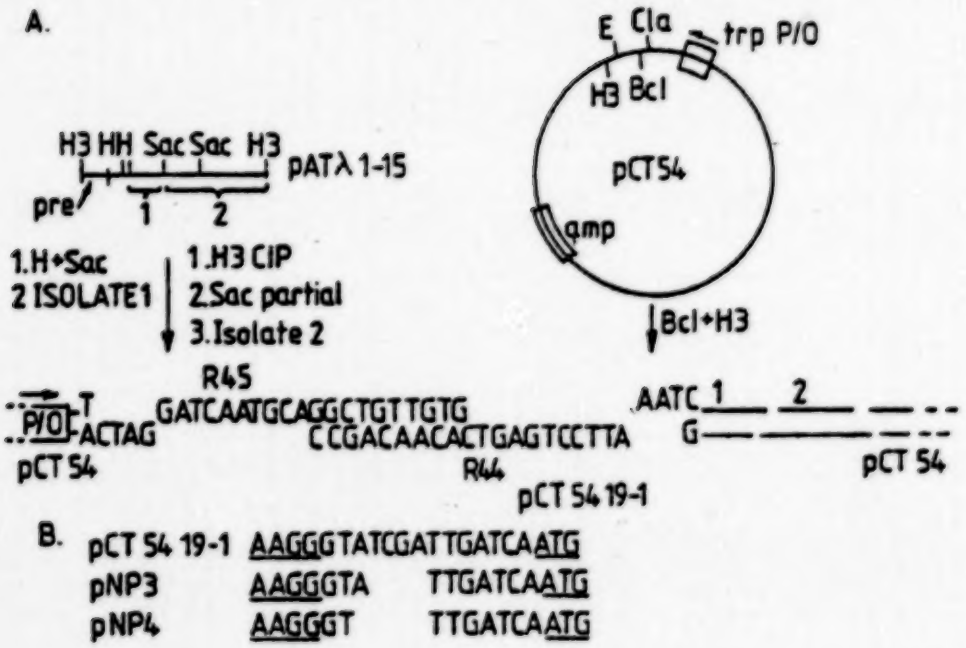


FIG. 1



B.

pCT 54 19-1	AAGGGTATCGATTGATCAATG	
pNP3	AAGGGTA	TTGATCAATG
pNP4	AAGGGT	TTGATCAATG

Legend. E = E corRI
H = HinfI
H3= Hind III

FIG. 2

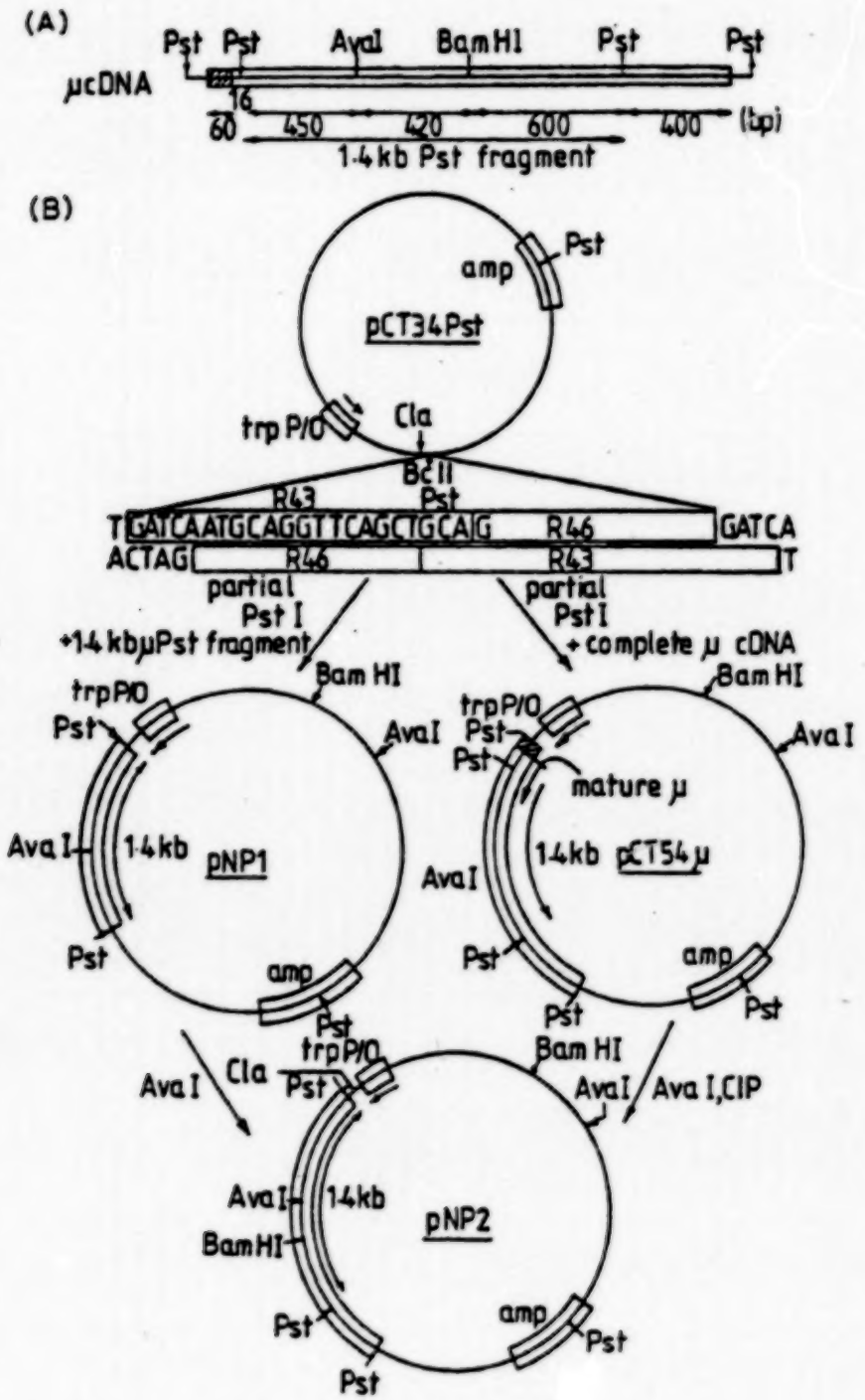

(A)
Pst Pst AvaI BamHI Pst Pst
μcDNA
16
60 450 420 600 400 (bp)
1.4kb Pst fragment
(B)
amp
Pst
pCT34Pst
trp P/O
Cla
BclI
Pst
R43
R46
GATCA
ACTAG
partial
Pst I
+1.4kbμPst fragment
+complete μ cDNA
Bam HI
trpP/O
Pst
AvaI
1.4kb
pNP1
mature μ
pCT54μ
Cla
AvaI, CIP
pNP2
BamHI

FIG. 3

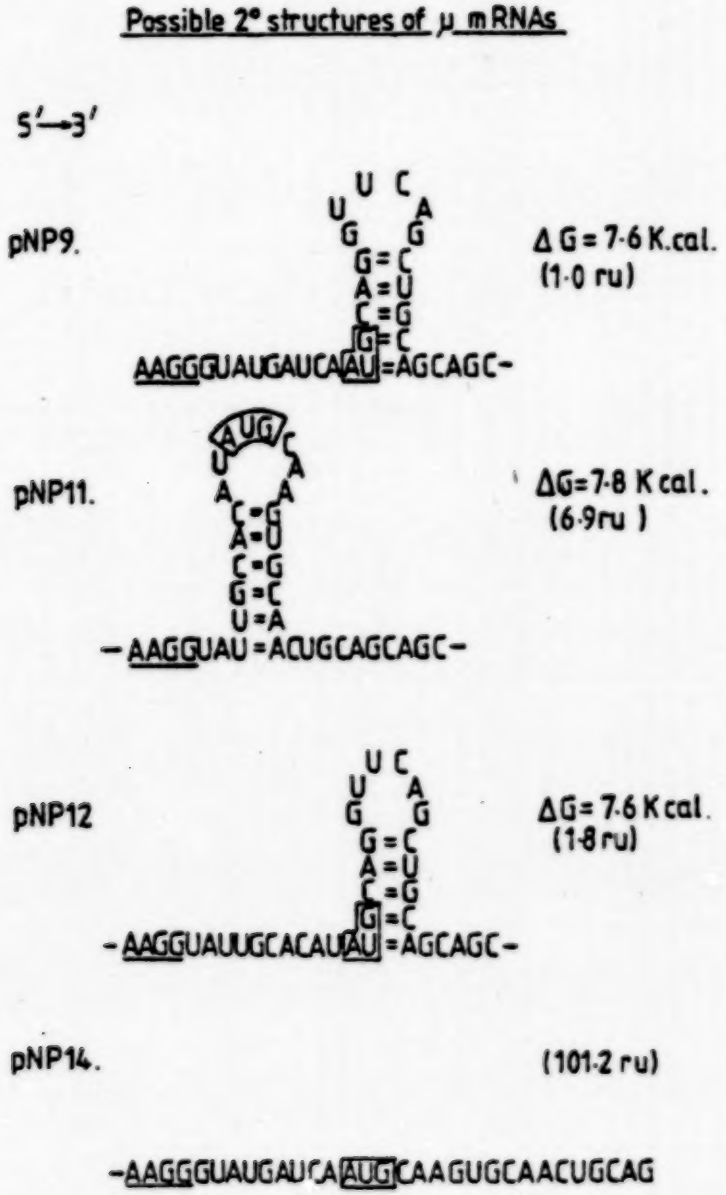

Possible 2° structures of μ mRNAs
5'→3'
pNP9.
ΔG = 7·6 K.cal.
(1·0 ru)
AAGGGUAUGAUCAAUG=AGCAGC-
pNP11.
ΔG=7·8 K cal.
(6·9ru)
-AAGGUAU=ACUGCAGCAGC-
pNP12
ΔG= 7·6 K cal.
(1·8 ru)
-AAGGUAUUGCACAUAUG=AGCAGC-
pNP14.
(101·2 ru)
-AAGGGUAUGAUCAAUGCAAGUGCAACUGCAG

FIG. 4

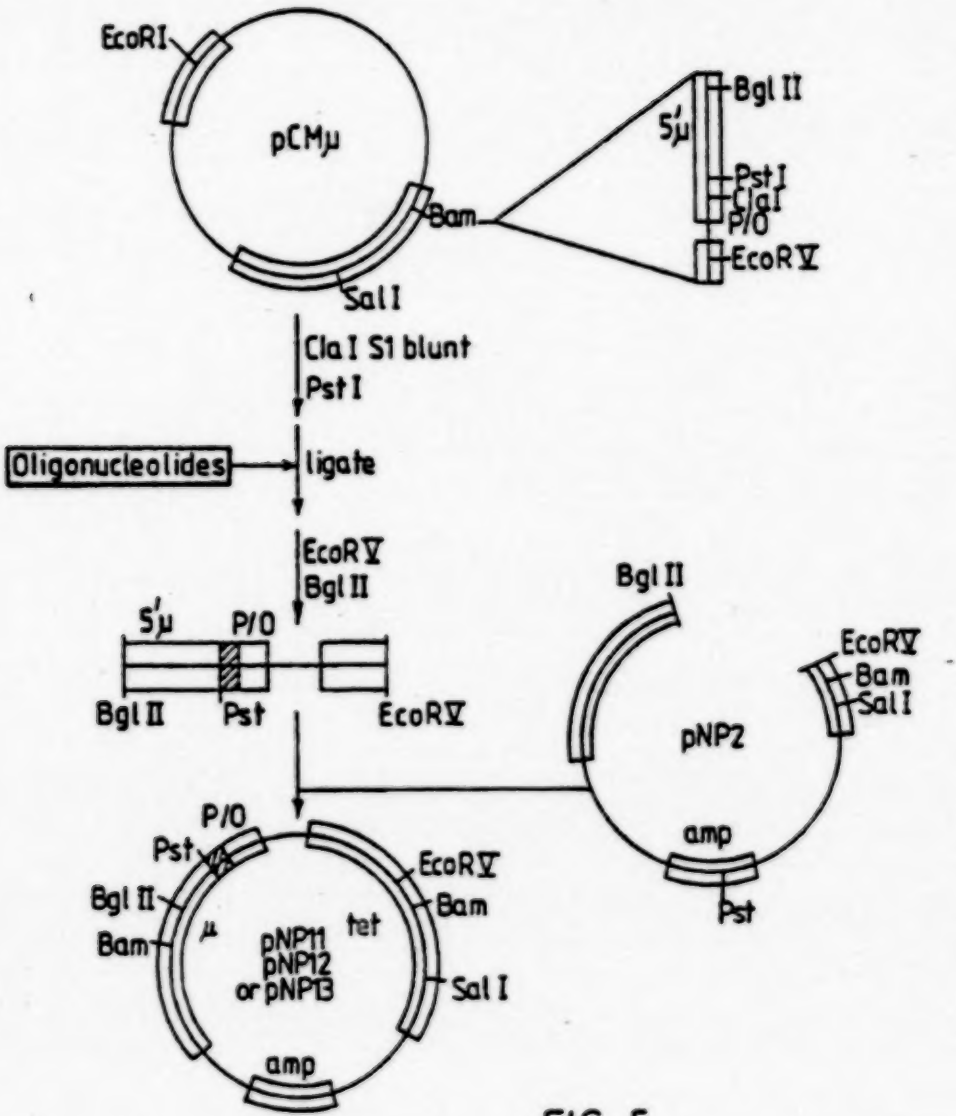

EcoRI
pCMμ
Bam
Sal I
5'μ
Bgl II
Pst I
Cla I
P/O
EcoR V
Cla I S1 blunt
Pst I
Oligonucleolides
ligate
EcoR V
Bgl II
5'μ
P/O
Bgl II
Pst
EcoR V
Bgl II
pNP2
EcoR V
Bam
Sal I
amp
Pst
P/O
Pst
Bgl II
Bam
μ
pNP11
pNP12
or pNP13
tet
EcoR V
Bam
Sal I
amp

FIG. 5

FIG. 6

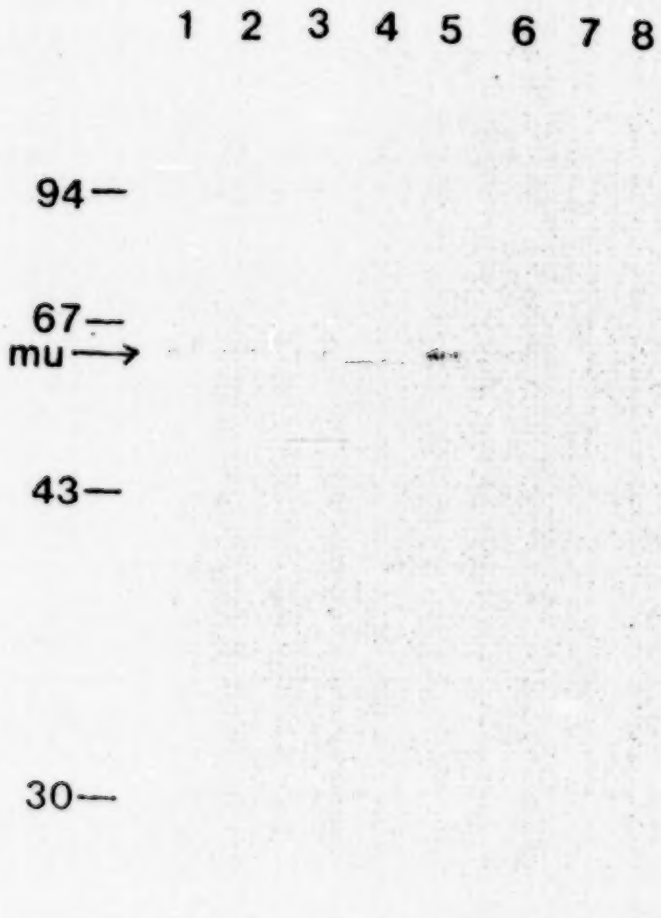


FIG. 7

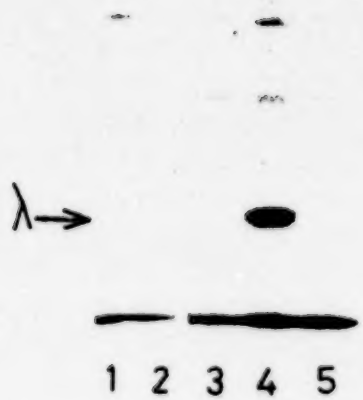


FIG. 8

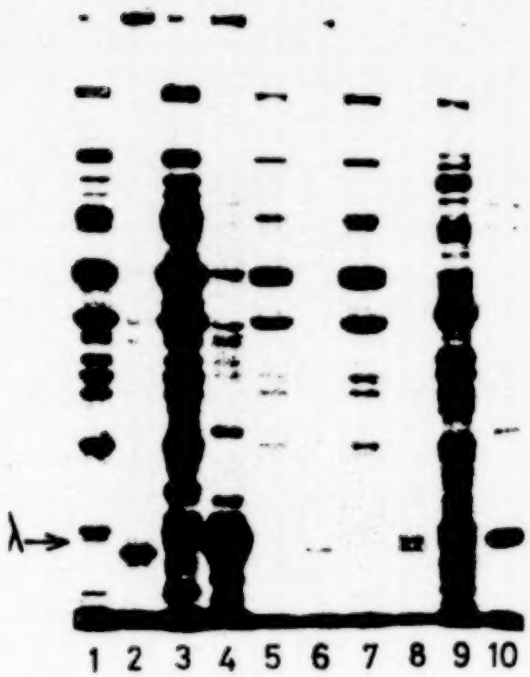


FIG. 9

FIG. 10

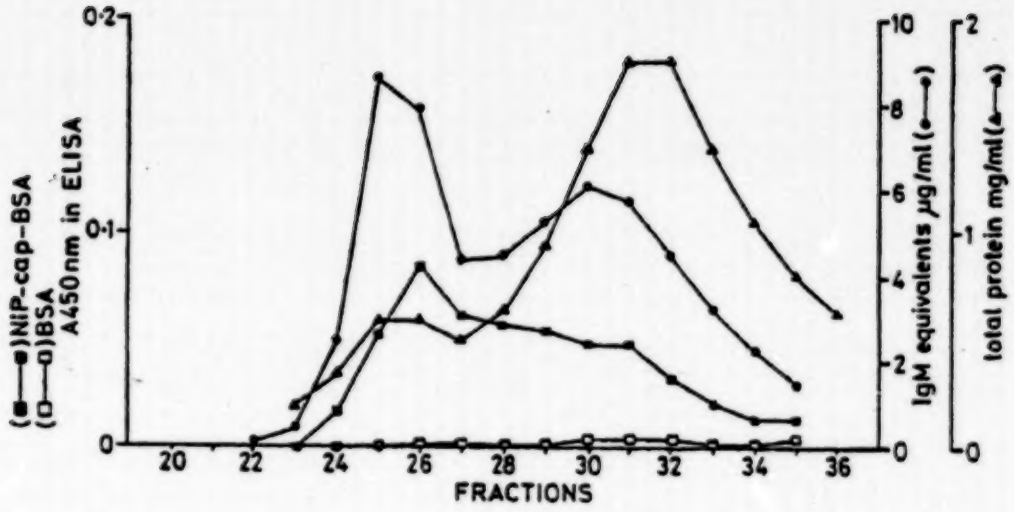

0·2
0·1
0
(■—■)NIP-cap-BSA
(□—□)BSA
A450nm in ELISA
10
8
6
4
2
0
IgM equivalents µg/ml (●—●)
2
1
0
total protein mg/ml (▲—▲)
20
22
24
26
28
30
32
34
36
FRACTIONS

FIG. 11

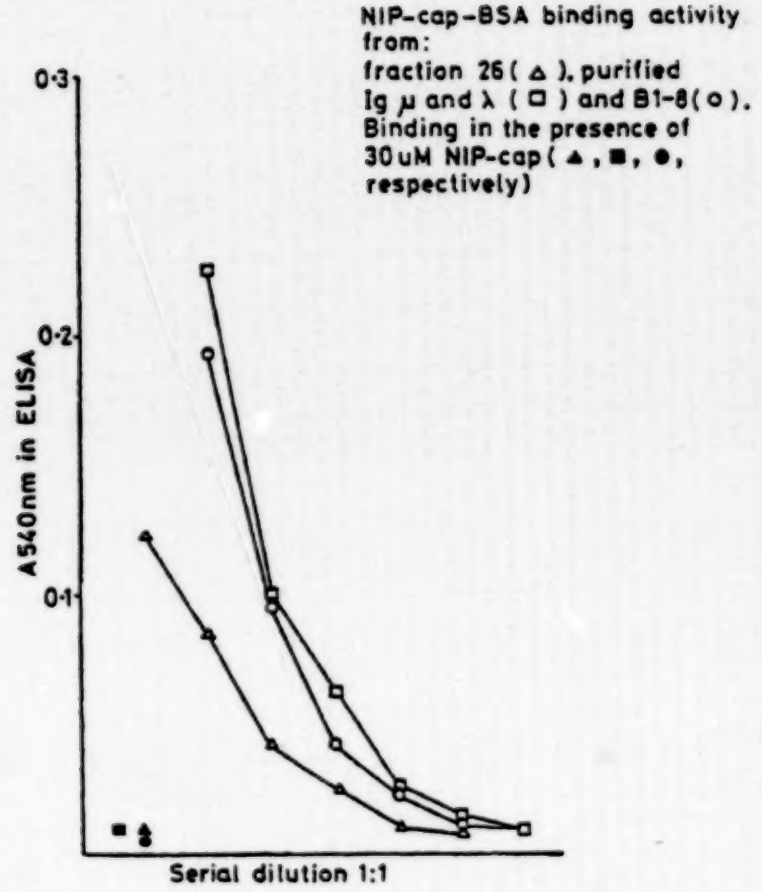
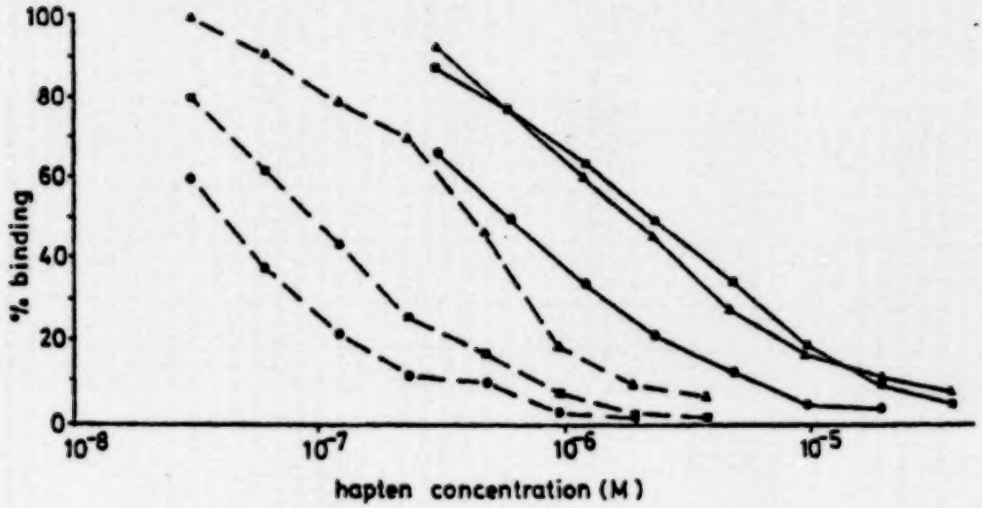

NIP-cap-BSA binding activity from:
fraction 26 (△), purified Ig μ and λ (□) and B1-8 (○).
Binding in the presence of 30 uM NIP-cap (▲ , ■ , ● , respectively)
0·3
0·2
0·1
A540nm in ELISA
Serial dilution 1:1

FIG. 12



Binding of antibodies to NIP-cap BSA B1-8 IgM (■), fraction 26 (▲), purified Ig μ or λ (●), in the presence of free NIP-cap (----) or NIP-cap (——).

FIG. 13

MULTICHAIN POLYPEPTIDES OR PROTEINS AND PROCESSES FOR THEIR PRODUCTION

This invention relates to multichain polypeptides or proteins and processes for their production in cells of host organisms which have been transformed by recombinant DNA techniques.

In recent years advances in molecular biology based on recombinant DNA techniques have provided processes for the production of heterologous (foreign) polypeptides or proteins in host cells which have been transformed with heterologous DNA sequences which code for the production of these products.

Theoretically, the recombinant DNA approach may be applied to the production of any heterologous polypeptide or protein in a suitable host cell, provided that appropriate DNA coding sequences can be identified and used to transform the host cell. In practice, when the recombinant DNA approach was first applied to the production of commercially useful products, its application for the production of any specified polypeptide or protein presented particular problems and difficulties, and the success of applying this approach to the production of any particular polypeptide or product was not readily predictable.

However, a large number of heterologous single chain polypeptides or proteins have now been produced by host cells transformed by recombinant DNA techniques. Examples of such heterologous single chain polypeptides or proteins include human interferons, the A and B chains of human insulin, human and bovine growth hormone, somatostatin, calf prochymosin and urokinase. Such transformed host cells provide a reproducible supply of authentic heterologous polypeptiede or protein which may be produced on an industrial scale using industrial fermentation technology.

It should be pointed out that some of these polypeptides, for instance urokinase, after secretion by a host cell appear as two chain molecules. However, in such cases, the molecule is synthesised by the host cell as a single chain polypeptide, coded for by a single DNA sequence, which is cleaved in the host cell subsequent to synthesis to form the two chain structure.

It is known that in both human and animal systems there are a number of polypeptides or proteins which have multichain structure in which the chains are not derived from the cleavage of a single chain polypeptide coded for by a single DNA sequence. In such cases, the gene for each of the chains may be located at different points on the same chromosome or even on different chromosomes. In these cases, the polypeptide chains are synthesised separately and then assembled into the complete molecule subsequent to synthesis. Heretofore, no such multichain polypeptide or protein has been produced by recombinant DNA techniques from a single host cell.

A particular example of a class of such multichain polypeptides or proteins is the immunoglobulins.

Immunoglobulins, commonly referred to as antibodies, are protein molecules produced in animals by B-lymphocyte cells in response to challenge with foreign antigenic agents, such as bacteria, viruses and foreign proteins. The immunoglobulins comprise a crucial part of the immune systems of humans and animals. The immunoglobulins recognise specific parts of the foreign agents and bind onto them. The specific parts are usually known as antigenic determinants or antibody binding sites. A given foreign agent is likely to have a number of different antigenic determinants.

A typical immunoglobulin (Ig) molecule is shown in FIG. 1 of the accompanying drawings, to which reference is now made. The Ig molecule comprises two identical polypeptide chains of about 600 amino acid residues (usually referred to as the heavy chains H), disulphide bonded to each other, and two identical shorter polypeptide chains of about 220 amino acid residues (usually referred to as the light chains L), each light chain being disulphide bonded to one end of each heavy chain as shown.

When the Ig molecule is correctly folded, each chain is formed into a number of distinct globular areas, usually known as domains, joined by a more linear polypeptide chain. The light chains have two such domains, one of which is of variable sequence V_L, and the other of which is of constant sequence C_L. The heavy chains have a single variable domain V_H adjacent the variable domain V_L of the light chain, and three or four constant domains $C_{H1\text{-}3 \text{ or } 4}$.

The variable domains on both the heavy and the light chains each contain three hypervariable regions (HV1-3) which, when the Ig molecule is correctly folded, are located adjacent one another and form the antigen binding site. It is this area which recognises and binds to the antigenic determinant for which the Ig molecule is specific.

The constant domains of the Ig molecule do not take part in the binding to the antigenic determinant, but mediate the actions triggered by the binding of the Ig molecule to the antigenic determinant. It is believed that this triggering is caused by an allosteric effect induced by the binding of the Ig molecule to the antigenic determinant. The constant domain may enable the Ig molecule to fix complement or may cause mast cells to release histamine.

Ig's may be categorised by class or subclass, depending on which of a number of possible heavy chain constant domains they contain, there being eight such possible heavy chains in mice. Thus, for instance, and Ig molecule with a μ heavy chain belongs to the class IgM, and one with a γ_1 heavy chain to the class IgG_1.

Ig's may also contain one of two light chains, designated as κ and λ light chains, which have different constant domains and different sets of variable domains.

The structure of the Ig molecule and the location of the genes coding for the various domains thereof are discussed more fully by Early and Hood, in Genetic Engineering, Principles and Methods, Vol. 3, pages 153-158 (edited by Setlow and Hollaender, Plenum Press).

It is known that Ig molecules on digestion with selected enzymes can produce a number of immunologically functional fragments. Two such fragments are known as the F_{ab} and $(F_{ab}')_2$ fragments. The F_{ab} fragment comprises one light chain linked to the V_H and C_{H1} domains of a heavy chain as shown in FIG. 1. The $(F_{ab}')_2$ fragment consists essentially of two F_{ab} fragments linked together by a small additional portion of the heavy chains as shown in FIG. 1. These fragments and other similar fragments can be of use in various tests and diagnostic and medical methods.

The principle method employed for the production of Ig's involves the immunisation of susceptible animals with the antigenic agent to provide an immune reaction. Generally the animal is immunised a second time to

improve the yield of Ig. The animal is then bled and the Ig is recovered from the serum.

However, the product of this method is not a homogeneous protein. The animal will produce Ig of different classes and also Ig specific for each of the different antigenic determinants on the antigenic agent, and its blood will therefore contain a heterogeneous mixture of Ig's. Obtaining a specific Ig of particular class and desired specificity from such a mixture requires very difficult and tedious purification procedures.

Recently, it has become possible to produce a homogeneous Ig of a single class and a single specificity by a technique first described by Kohler and Milstein (Nature, 256, 495–479, 1975). The technique involves the fusion of single Ig-producing parent cells with cancer cells to produce a monoclonal hybridoma cell which produces the Ig. Ig produced by this technique is usually known as monoclonal antibody. The nature of the monoclonal Ig is determined by the class and specificity of the Ig produced by the parent cell.

Recently attempts have been made to use recombinant DNA techniques to produce fragments of Ig molecules. For instance, Amster et al. (Nucleic Acid Research, 8, No. 9, 1980, pp 2055 to 2065) disclose the cloning of double stranded cDNA sequences encoding for a mouse Ig light chain into a plasmid. An E. Coli strain transformed by the plasmid synthesised a protein thought to comprise the complete constant domain of the light chain and about 40 amino acid residues of its variable region.

Kemp and Cowman (Proc. Natl. Acad. Sci. USA, 78, 1981 , pp 4520 to 4524) disclose the cloning of cDNA sequences encoding for mouse heavy chain fragments and the transforming of an E. Coli strain which then synthesised heavy chain polypeptide fragments.

In both these cases, the polypeptides were produced as fusion proteins, in which the fragments of the Ig polypeptides were fused with additional non-Ig polypeptide sequences, and the incomplete variable domains. Thus, the polypeptide chains produced in these studies were not immunologically functional polypeptides as they were incapable of combining with complementary heavy or light chains to provide Ig molecules having intact antigen binding sites and immunological function.

Research studies have also been carried out in mammalian systems. For instance, Falkner and Zachau (Nature, 298, 1982, pp 286 to 288) report the cloning of cDNA sequences encoding for mouse light chains into a plasmid which was used to transfect genomic eukaryotic cells which could then transiently synthesise light chains.

Rice and Baltimore (Proc. Natl. Acad. Sci. USA, 79, 1982, pp 7862 to 7865) report on the transfection of a functionally rearranged K light chain Ig gene into a murine leukemia virus-transformed lymphoid cell line. The cell line is then able to express the gene continuously. In both these cases, the K genes used to transfect the mammalian cells were obtained from myeloma cells and the K polypeptides produced were of indeterminate immunological function.

A further approach is exemplified in a series of papers by Valle et al. (Nature, 291, 1981, 338–340; Nature, 300, 1982, 71–74and J. Mol. Biol., 160, 1982, 459–474), which describe the microinjection of mRNAs encoding for heavy or light chains of Ig isolated from a mouse myeloma line into oocytes of Xenopus laevis. Under certain conditions complete Ig molecules were formed. However, the mRNAs were obtained from myeloma cells and the Ig molecules were of indeterminate immunological function.

It can thus be seen that hitherto it has not been possible to produce functional Ig by recombinant DNA technology.

According to a first aspect of the present invention, there is provided a process for producing a heterologous multichain polypeptide or protein in a single host cell, which comprises transforming the host cell with DNA sequences coding for each of the polypeptide chains and expressing said polypeptide chains in said transformed host cell.

According to a second aspect of the present invention, there is provided a heterologous multichain polypeptide or protein produced by recombinant DNA technology from a single host cell.

The present invention is of particular, but not exclusive, application in the production of Ig molecules and immunologically functional Ig fragments of the type referred to above. However, it will be appreciated that the present invention can be applied to the production of other multichain polypeptides or proteins.

In relation to the product of Ig molecules according to the invention it will be appreciated that, in order to produce a functional molecule, the DNA sequences used to transform the host cell will need to encode for at least the V_L and V_H domains of an Ig molecule. Moreover, these domains will need to be complementary so that when the two polypeptide chains fold together they form an antigen binding site of predetermined specificity.

Preferably, the Ig molecule or fragment include a complete light chain and at least the C_{H1} domain in addition to the V_H domain of the heavy chain. Most preferably the Ig molecule is intact.

It has also been shown by the present applicants that it is now possible to produce individual heavy and light chains having intact variable domains. This has not previously been possible. Therefore, according to a third aspect of the present invention there is provided as a product of recombinant DNA technology an Ig heavy or light chain or fragment thereof having an intact variable domain.

Advantageously, the Ig molecule or functional fragment thereof according to the present invention has a variable region (formed by the V_L and V_H domains) which defines a binding site for an antigenic determinant of clinical or industrial importance. The DNA coding sequences necessary to produce such a molecule may be derived from naturally occuring or hybridoma (monoclonal)Ig-producing cells with the desired specificity.

The constant domains of the Ig molecule or fragment, if present, may be derived from the same cell line as the variable region. However, the constant domains may be specifically altered, partially or completely omitted, or derived from a cell line producing a different class of Ig to provide Ig molecules or fragments having desired properties.

For example, an Ig molecule may be produced having variable domains (V_H and V_L) idential with those from a monoclonal antibody having a desired specificity, and constant domain(s) from a different monoclonal antibody having desired properties, for instance to provide human compatibility or to provide a complement binding site.

Such alterations in the amino acid sequence of the constant domains may be achieved by suitable mutation or partial synthesis and replacement or partial or complete substitution of appropriate regions of the corresponding DNA coding sequences. Substitute constant domain portions may be obtained from compatible recombinant DNA sequences.

The invention may be utilised for the production of Ig molecules or fragments useful for immunopurification, immunoassays, cytochemical labelling and targetting methods, and methods of diagnosis or therapy. For example, the Ig molecule or fragment may bind to a therapeutically active protein such as interferon or a blood clotting factor, for example Factor VIII, and may therefore be used to produce an affinity chromatorgraphy medium for use in the immunopurification or assay of the protein.

It is also envisaged that the Ig molecule may be synthesised by a host cell with another peptide moiety attached to one of its constant domains. Such a further peptide moiety may be cytotoxic or enzymatic. Alternatively, the moiety may be useful in attaching the Ig molecule to a biological substrate, such a cell or tissue, or to a non-biological substrate, such as a chromatography medium. Such a peptide moiety is herein referred to as a structural peptide moiety.

It is further envisaged that cytotoxic, enzymic or structural peptide moieties could be attached to the Ig molecule by normal peptide chemical methods, as are already known in the art, rather than by being synthesised with the Ig molecule.

The Ig molecule or fragment may also comprise a therapeutic agent in its own right. For instance, an Ig molecule or fragment specific for D blood group antigen may be useful for the prevention of haemolytic disease of the new born.

Any suitable recombinant DNA technique may be used in the production of the multichain polypeptides or proteins of the present invention. Typical expression vectors such as plasmids are constructed comprising DNA sequences coding for each of the chains of the polypeptide or protein.

It will be appreciated that a single vector may be constructed which contains the DNA sequences coding for more than one of the chains. For instance, the DNA sequences coding for Ig heavy and light chains may be inserted at different positions on the same plasmid.

Alternatively, the DNA sequence coding for each chain may be inserted individually into a plasmid, thus producing a number of constructed plasmids, each coding for a particular chain. Preferably the plasmids into which the sequences are inserted are compatible.

The or each constructed plasmid is used to transform a host cell so that each host cell contains DNA sequences coding for each of the chains in the polypeptide or protein.

Suitable expression vectors which may be use for cloning in bacterial systems include plasmids, such as Col E1, pcR1, pBR322, pACYC 184 and RP4, phage DNA or derivatives of any of these.

For use in cloning in yeast systems, suitable expression vectors include plasmids based on a 2 micron origin.

Any plasmid containing an appropriate mammalian gene promoter sequence may be used for cloning in mammalian systems. Such vectors include plasmids derived from, for instance, pBR322, bovine papilloma virus, retroviruses, DNA viruses and vaccinia viruses.

Suitable host cells which may be used for expression of the heterologous multichain polypeptide or protein include bacteria, such as *E. coli* and *B. subtilis*, Streptomyces, yeasts, such as *S. cervisiae*, and eukaryotic cells, such as insect or mammalian cell lines. Examples of suitable bacterial host cells include *E. Coli* HB 101, *E. Coli* X1776, *E. Coli* X2882, *E. Coli* PS 410, *E. Coli* MRC 1, *E. Coli* RV308, *E. Coli* E103S and *E. Coli* B.

The present invention also includes constructed expression vectors and transformed host cells for use in producing the multichain polypeptides or proteins of the present invention.

After expression of the individual chains in the same host cell, they may be recovered to provide the complete multichain polypeptide or protein in active form, for instance to provide an Ig molecule of predetermined immunological function.

It is envisaged that in preferred forms of the invention, the individual chains will be processed by the host cell to form the complete polypeptide or protein which advantageously is secreted therefrom.

However, it may be that the individual chains may be produced in insoluble or membrane-bound form. It may therefore be necessary to solubilise the individual chains and allow the chains to refold in solution to form the active multichain polypeptide or protein. A suitable procedure for solubilising polypeptide chains expressed in insoluble or membrane-bound form is disclosed in our copending application No. (Protein Recovery, Agent's Ref. GF 402120 and 402121).

It will be appreciated that the present application shows for the first time that it is possible to transform a host cell so that it can express two or more separate polypeptides which may be assembled to form a complete multichain polypeptide or protein. There is no disclosure or suggestion of the present invention in the prior art, which relates solely to the production of a single chain heterologous polypeptide or protein from each host cell.

The present invention will now be described, by way of example only, with reference to the accompanying drawings, in which:

FIG. 1. shows a diagrammatic representation of a typical intact Ig molecule;

FIG. 2 shows the construction of plasmids for the direct synthesis of a λ light chain in *E. Coli;*

FIG. 3 shows the construction of plasmids for the direct synthesis of a μ heavy chain in *E. Coli;*

FIG. 4 is a diagrammatic representation of μ*m*RNA sequences around the initiation codon;

FIG. 5 shows the construction of plasmids having altered secondary structure around the initiation codon;

FIG. 6 is a polyacrylamide gel showing expression and distribution of μ protein from *E. Coli* B;

FIG. 7 is a polyacrylamide gel showing pulse chase autoradiograms of μ protein in *E. Coli* B and in *E. Coli* HB101;

FIG. 8 is a polyacrylamide gel showing the results of λ gene expression in *E. Coli;*

FIG. 9 is a polyacrylamide gel showing the distribution of recombinant λ light chain polypeptide between the soluble and insoluble cell fractions;

FIG. 10 is a polyacrylamide gel showing expression and distribution of λ protein from *E. Coli* E103S;

FIG. 11 shows the results of the fracitonation of μ and λ protein expressed by *E. Coli* B on DEAE Sephacel;

FIG. 12 shows the specific hapten binding of reconstituted Ig molecules; and

FIG. 13 shows the heteroclitic nature of the hapten binding of the reconstituted Ig molecules.

In the following examples, there is described the production of Ig light and heavy chain polypeptides derived from monoclonal antibodies which recognise and bind to the antigenic determinant 4-hydroxy-3-nitrophenyl acetyl (NP), using *E. coli* and *S. cerevisiae* as the host cells Recombinant DNA techniques were used to enable the host cells to express both the polypeptide chains.

It will be appreciated that the invention is not limited to the specific methods and construction described hereafter.

Construction of Lambda Light Chain Expression Plasmid

FIG. 2, to which reference is now made, shows schematically the method used to construct a λ light chain expression plasmid.

It was decided to express the lambda gene in *E. coli* by direct expression of the gene lacking the eucaryotic signal peptide but containing a methionine initiator residue at the amino-terminus (met-lambda). The approach used for bacterial synthesis of met-lambda was to reconstruct the gene in vitro from restriction fragments of a cDNA clone and to utilise synthetic DNA fragments for insertion into the bacterial plasmid pCT54 (Emtage et al., proc. Natl. Acad. Sci. USA., 80, 3671 to 3675, 1983). This vector contains the *E. coli* trp promoter, operator and leader ribosome binding site; in addition 14 nucleotides downstream of the ribosome binding site is an initiator ATG followed immediately by $EcoR_I$ and HindIII sites and the terminator for *E. coli* RNA polymerase from bacteriophage T7.

As a source of light chain we used a plasmid pABλ-1-15 which contains a full-length λ_I light chain cDNA cloned into the PstI site of pBR322. This λ_I light chain is derived from a monoclonal antibody, designated S43, which binds to 4-hydroxy-3-nitrophenylacetyl (NP) haptens.

In order to create a HindIII site 3' to the end of the lambda gene for insertion into the HindIII site of pCT54, the cDNA was excised from pAB λ 1-15 using PstI. The cohesive ends were blunt ended using the Klenow fragment of DNA polymerase and synthetic HindIII linker molecules of sequence 5'-CCAAGCTTGG-3' ligated. The DNA was digested with HindIII and the 850bp lambda gene isolated by gel electrophoresis and cloned into HindIII cut pAT153 to yield plasmid pAT λ 1-15. The 3' end of the lambda gene was isolated from pAT λ 1-15 by HindIII plus partial SacI digestion as a 630bp SacI-HindIII fragment (2 in FIG. 2). The HindIII cohesive end was dephosphorylated by calf intestinal alkaline phosphatase during isolation of the fragment to prevent unwanted ligations at this end in subsequent reactions.

A HinfI restriction site is located between codons 7 and 8 and the lambda sequence. The 5' end of the lambda gene was isolated as a 148bp HinfI to sacI fragment (1 in FIG. 2).

Two oligodeoxyribonucleotides were designed to restore condons 1-8, and to provide an initiator ATG as well as BclI and HinfI sticky ends. The two chemically synthesised oligonucleotides made to facilitate assembly of the gene had the sequences:

R45 5'-pGATCAATGCAGGCTGTTGTG 3'

R44 3' CCGACAACACTGAGTCCTTAp- 5'

pCT54 was cut with both BclI and HindIII and the resulting linear molecules isolated, mixed together with the two oligodeoxyribonucleotide linkers R44 and R45 and both fragments 1 and 2, and ligated using T4 ligase (FIG. 2). The mixture was used to transform *E. coli* DH1 to ampicillin resistance. Recombinant clones in pCT54 were identified by hybridisation of DNA from replica plated colonies on nitrocellulose to a nick-translated probe derived from the pAT λ 1-15 insert.

A clone was identified which hybridised to lambda cDNA and also showed the predicted restriction fragment pattern. This plasmid (designated pCT54 19-1) was sequenced from the ClaI site and shown to have the anticipated sequence except that there was a mutation of the fourth codon from CTG to ATG, changing the amino acid at this point from valine the methionine.

The sequence in this area was:

```
... GATTGATCA.ATG.CAG.GCT.GTT.ATG.ACT.CAG.GAA.TCT.GCA.CTC.ACC.ACA.TCA
      met  gln  ala  val  met  thr  gln  glu  ser  ala  leu  thr  thr  ser
```

The restriction enzyme sites in pCT54 between the Shine and Dalgarno sequence (AAGG), which is important for ribosome binding, and the ATG allow for the adjustment of the SD-ATG distance, an important parameter in determining expression rates. The SD-ATG distance was reduced by cutting the plasmid with ClaI or BclI and creating blunt ended species by digestion with S_1 nuclease. 2 μg of ClaI cut DNA was digested with 200 units of S_1 nuclease for 30 minutes at 30° using standard buffer conditions. The solution was deproteinised with phenol and the DNA recovered by ethanol precipitation. This DNA on religation with T4 DNA ligase and transformation into *E. coli* strain HB101 gave rise to a number of plasmids which had lost the ClaI or BclI site.

The plasmids which had lost their ClaI site were sequenced in the region surrounding the initiator ATG.

```
... AAGGGTATTGATCAATG CAG ... plasmid pNP3
      SD              met glu

... AAGGGTTTGATCAATG CAG      plasmid pNP4
      SD              met glu
```

In order to achieve high level expression a number of other approaches were followed. Firstly, a series of constructs were obtained which had increasing amounts of the 3' untranslated region of the cDNA removed by Bal 31 exonuclease. Secondly, a high copy number plasmid containing λcDNA was constructed. This plasmid contained a par function (Meacock, P. A. and Cohen, S. N., Cell, 20, 529-542, 1980) as well as being present in high copy number. Thirdly, the pNP3 plasmid was transformed into a number of protease-deficient strains or into HB101 in conjunction with a protease deficient dominant acting plasmid (Grossman, A. D. et al, Cell, 32, 151-159, 1983).

Construction of μ Heavy Chain Expression Plasmid

The full-length μ heavy chain cDNA derived from the NP binding monoclonal antibody B1-8 had been cloned into the PstI site of pBR322 yielding a plasmid designated pABμ-11 (Bothwell et al, Cell, 24, 625-637, 1981). In order to achieve high level expression, the μ cDNA minus the eukaryotic leader was reconstructed into pCT54. The construction of the μ heavy chain expression plasmid is shown diagramatically in FIG. 3. Two chemically synthesised oligonucleotides were made to facilitate this. These have the following sequences:

R43 5' GATCAATGCAGGTTCAGCTGCA 3'

R46 3' TTACGTCCAAGTCG 5'

These were ligated into BclI cut pCT54 using T4 DNA ligase and the resulting plasmid designated pCT54 Pst. The linkers were designed to replace the sequence in pCT54 between the BclI site and the ATG, to provide an internal PstI site and to recreate the sequence from pABμ-11 5' to the PstI site up to codon +1. pCT54 Pst was cut briefly was PstI, treated with alkaline phosphatase and full length DNA glass isolated from a 1% agarose gel. Similarly pABμ-11 was briefly cut with PstI and the full length μ insert isolated following agarose gel electrophoresis. The μ cDNA was ligated with the full length pCT54Pst fragment using T4 DNA ligase under standard conditions and a plasmid designated pCT54μ was identified which by restriction enzyme analysis was shown in contain a full-length μ insert. The plasmid was sequenced around the 5' linker region and was found to have the anticipated sequence:

5' . . .
TGATCAATGCAGGTTCAGCT-
GCAGGGGGGGGATGGATGGAG . . . 3',

demonstrating that it was, indeed, a full length clone. A complete PstI digest of pCT54μ liberated a 1.4 Kb fragment which was purified by 0.8% agrose gel electrophoresis and glass powder isolation. This was ligated with PstI cut pCT54 Pst (see above) using T4 DNA ligase under standard conditions, followed by transformation into HB101. A plasmid designated pNP1 was isolated which was shown by restriction endonuclease pattern analysis to contain the 1.4 Kb μcDNA fragment in an appropriate orientation (FIG. 3). pNP1 was a plasmid which consisted of an appropriate 5' end for expression, whilst pCT54 contained an appropriate 3' end. The full length gene was reconstructed into pCT54 by cutting both pNP1 and PCT54μ with AvaI which cuts once in the plasmid and once in the μ gene. Both digests were run on a 1% agrose gel and the 1.9 kb fragment from pNP1 and the 3.65 kb fragment from pCT54μ isolated. Following alkaline phosphatase treatment of the 3.65 pCT54μ fragment, the two pieces of DNA were ligated to each other and transformed into HB101. A plasmid designated pNP2 was identified which demonstrated the correct restriction endonuclease pattern. It was sequenced in the area surrounding the initiator ATG and found to have the anticipated sequence:

5' . . .
TTGATCAATGCAGGTTCAGCTGCAG-
CAGCCTGGGGCTGAGCTTGTGAAG . . . 3'

The vector pCT54 had been constructed to include two restriction sites (BclI and ClaI) between the S-D sequence (AAGG) and the initiation codon so that the distance between these sequence elements could be varied. As most *E. coli* mRNAs have 6-11 nucleotides between the S-D sequence and the AUG, the distance in pNP2 was reduced by modification at the ClaI site. pNP2 was cut with ClaI and incubated with S1. The amount of S1 nuclease was adjusted so that some DNA molecules would lose 1-2 extra base pairs as a result of 'nibbling' by the enzyme. This DNA on religation with T4 DNA ligase and transformation into *E. coli* strain HB101 gave rise to a number of colonies harbouring plasmids which had lost the ClaI site. The sequences around the initiation codon of four plasmids pNP223, pNP261, pNP9 and pNP282 were determined and are given in Table 1, part A below.

TABLE 1

		S—D to ATG distance (no. of residues)	Sequence (5' - 3') Clot	Expression:(mu)(ru) expts. A	B	C
(A)	pNP2	14	—AAAAAGGGTATCGATTGATCA[ATG]CAGGTTCAG—	0.7	0.6	0.8
S1 derivatives of pNP2	pNP223	12	—AAAAAGGGTATATTGATCA[ATG]CAGGTTCAG—	—	0.7	0.6
	pNP261	10	—AAAAAGGGTATTGATCA[ATG]CAGGTTCAG—	—	1.1	1.1
	pNP282	7	—AAAAAGGGTGATCA[ATG]CAGGTTCAG—	—	0.7	0.6
	pNP9	9	—AAAAAGGGTATGATCA[ATG]CAGGTTCAG—	1.0	1.0	1.0
(B)	pNP11	11	—AAAAAGGGTATTGCACAT[ATG]CAAGTGCAA—	5.3	6.3	9.1
oligo constructs	pNP12	11	—AAAAAGGGTATTGCACA[ATG]CAGGTTCAG—	1.8	2.3	1.3

TABLE 1-continued

	S—D to ATG distance (no. of residues)	Sequence (5' - 3') Clot	Expression:(mu)(ru) expts. A	B	C
pNP14	9	—AAAAAGGGTATGATCA[ATG]CAAGTGCAA—	72.2	106.7	124.6
pNP8	—	—	77.0	53.4	145.5
pCT70	—	—	0	0	0

The expression of these constructs in *E. coli* B was examined in three experiments, and concentrations of μ protein were determined by ELISA as relative units (ru), a measure of μ concentration normalised to pNP9. The results are shown in Table 1.

Inductions were carried out by resuspending cells at 1:50 dilution from overnight cultures into induction medium, consisting of: KH_2PO_4 (3 g/l), Na_2HPO_4 (6 g/l), NaCl (0.5 g/l), Difco vitamin assay casamino acids (30 g/l), NH_4Cl (4 g/l), glycerol (16 g/l), proline (0.5 g/l), Difco yeast extract (1 g/l), $CaCl_2.6H_2O$ (0.022 g/l), $MgSO_4.7H_2O$ (0.025 g/l), thiamine (0.01 g/l) and carbenicillin (0.1 g/l). Cultures were shaken at 37° C. and cells harvested by centrifugation.

ELISA assays were performed essentially as described by Patel et al. (Nucleic Acid Research, 10, 5605 to 5620, 1982), using affinity purified goat anti-mouse IgM (Tago) and affinity purified goat anti-mouse IgM (Tago) conjugated peroxidase, with 3,3',5,5' tetramethylbenzidine (Miles) as a substrate. Bacterial cell pellets were boiled for 2 minutes in 9M urea (BRL) and loaded directly into the ELISA, such that all wells contained 2.25M urea, 10 mM Tris-HCl pH 7.8 150 mM NaCl, 0.05% NP40 and 0.5% casein (Hammarsten, B. D. H.).

Altering the S-D to ATG distance with nuclease S1 was found to increase the level of μ expression, relative, to the parental plasmid pNP2, but only to a small extent (compare pNP9). The optimal S-G to AUG distance was found to be 9–10 nucleotides, as found in pNP9 and pNP261 respectively.

Secondary Structure Analysis

Hairpin loops were identified by use of the computer programme HAIRGU developed by Dr. Roger Staden. The ΔG values were calculated as described by Trioco et al. (Nature NB, 246, 40–41, 1973).

Analysis of the potential secondary structure of μ mRNA encoded by pNP2 and pNP9 (one of the S1 derivatives of pNP2) revealed an extensive array of possible hairpin loops. Attention was focused on the region of this mRNA containing the ribosome binding site since the sequestering of S-D sequences and initiation codons into secondary structure may inhibit the initiation of translation. It was found that a hairpin loop including the U and G of the initiation codon could be formed (see FIG. 4). This hairpin loop is formed entirely within coding sequences and has a $\Delta G = -7.6$ kcal. No secondary structures were found which buried the S-D sequence, and none which mutually excluded the hairpin loop described above, and which had a lower ΔG.

To test whether expression of μ protein could be influenced by changes designed to alter potential secondary structure in the ribosome binding region, we used synthetic oligonucleotides to mutagenise this region of μ mRNA. The plasmids were constructed by insertion of a pair of oligonucleotides between the blunted Cla I site and the 5' Pst site of μ in pNP2 (see FIG. 5). The cloning strategy used to accomplish this, however, was multi-staged due to the presence of four Pst sites in pNP2. The vector pACYC184CM, which lacks Cla I and Pst I sites, was first constructed by cutting pACYC184 at its unique Cla I site, 'filling-in' the cohesive termini using T4 DNA polymerase and religating. Next, PACYC184CM was cut with BamHI, and the BamHI fragment from pNP2, bearing the 5'μ sequence, was inserted into it to form pCMμ (see FIG. 4). The vector pCMμ contained unique Cla I and Pst sites which were required for the oligonucleotide cloning (see FIG. 5).

pcMμ was cut with Cla I to completion, blunted with nuclease S1, and then cut with Pst I. The cut plasmid was purified by agarose gel electrophoresis and ligated separately with pairs of oligonucleotides, with the latter in a 100-fold molar excess. The pairs of oligonucleotides used were: R131 and R132 (for pNP11); R196 and R197 (for pNP12); and R202 and R203 (for pNP14). These have the sequences given below.

TGCACATATGCAAGTGCAACTGCA (R131),

GTTGCACTTGCATATGTGCA (R132),

GCTGAACCTGCATATGTGCA (R196),

TGCACATATGCAGGTTCAGCTGCA (R197),

GTTGCACTTGCATTGATC (R202),

GATCAATGCAAGTGCAACTGCA (R203).

The ligation mixture was then used to transform HB101. Recombinant clones were detected by colony hybridisation, on nitrocellulose filters, using one of each pair of oligonucleotides as a probe. Positive clones were sequenced and shown to have the correct nucleotide sequence. The μ sequences containing the cloned oligonucleotides were excised on Eco RV-Bgl II fragments. These were ligated to Eco RV-Bgl II cut pNP2 to reconstruct the full length μ genes (see FIG. 5). Three different plasmids were created by these procedures; pNP11, pNP12 and pNP14, having the nucleotide sequences shown in Table 1 above.

In the construct pNP11 the potentially deleterious hairpin loop of pNP2 was abolished by changing the residues in the degenerate position of three codons and by introducing a different S-D to AUG sequence. These changes allow the region between the S-D and AUG to base pair with the 5' end of μ coding sequence to form a hairpin loop of $\Delta G = -7.8$ kcal, approximately equal to that of pNP2, but leaving the AUG and S-D sequences exposed (see FIG. 4). The only other mutually exclusive hairpin loops are of $\Delta G = -2.3$, -3.6 and $+0.4$ kcal.

The construct pNP12 retains the S-D to AUG sequence of pNP11, but has the coding sequence of pNP2, such that the hairpin loop of pNP2 can form (see FIG. 4).

The final μ construct was pNP14, which had the S-D to AUG sequence of pNP9, but the 5' coding sequence of pNP11, so that the hairpin loop of pNP12 could not form. The construct pNB14 had no secondary structure burying the S-D or μ AUG.

μ Protein Expression from Constructs with Differing mRNA Secondary Structures

E. coli B strains containing plasmids pNP9, pNP11, pNP12 and pNP14 were grown in induction medium and samples removed for ELISA assay. The concentration of μ was increased 6–7 fold in *E. coli* B cells containing pNP11 relative to pNP9 (see Table 1, Part b). Plasmid pNP12, which has the S-D to AUG sequence of pNP11, but the coding sequence of pNP9, showed only a two-fold increase in level of μ expression compared to pNP9.

Plasmid pNP14, which has the S-D to AUG sequence of pNP9 but the 5'μcoding sequence of pNP11, and thus differed from pNP9 only in three residues, was found to express μ protein over 90 times that found in pNP9.

A plasmid, pNP8, has also been constructed, from which μ is expressed, also from the trp promoter, as part of a trpE-μ fusion protein containing the amino terminal 53 amino acids of trpE and the carboxyl terminal 503 amino acids of μ. The rationale for making this fusion gene was that μ would be translated from an effective bacterial ribosome binding site rather than from one of unknown efficiency. Then, by comparing the amounts of trpE-μ produced from pNP8 with those of μ produced from the different constructs, an estimate of relative ribosome binding efficiency may be made. When the trpE-μ gene in pNP8 was induced and the products quantitated by ELISA, it was found that the μ expression in strains containing pNP8 and pNP14 was very similar (see Table 1, part B). The ribosome binding site (RBS) sequences of pNP11, pNP12 and pNP14 were designed in order to test the hypothesis that sequestering of the initiation codon into secondary structure inhibits translation initiation, and to increase the rate of initiation by releasing the initiation codon. In pNP11, the RBS hairpin loop of pNP2 and pNP9 was abolished by changing the 5'μcoding sequence in the degenerate position of three codons. In addition, the S-D to AUG sequence was changed to allow the base-pairing of this region with the 5' coding sequence. The result of these changes is that the initiation codon is exposed in the loop of a hairpin with the S-D sequence at the base of the stem (see FIG. 4). This hairpin loop had a ΔG approximately equivalent to the ΔG of the pNP9 hairpin loop. Strains containing pNP11 produced 6–7 times as much μ as did those with pNP9. To test whether this was due to the change in S-D to ATG sequence characteristics, rather than to the liberation of the initiation codon, pNP12 was constructed as a control. The latter has the S-D to AUG sequence of pNP11, but retains the 5'μ coding sequence of pNP9, so that the RBS hairpin loop of pNP11 cannot form, but the potentially deleterious one of pNP9 can form. The level of μ expression from pNP12 was found to be increased by only two-fold relative to pNP9. This increase is probably due to an altered S-D to AUG distance, a U immediately 5' to the initiation codon, and the S-D to AUG sequence being G-poor each of which has been shown to be advantageous. It is therefore likely that the increased expression from pNP11 is due to a preferable mRNA secondary structure. The RBS hairpin loop of pNP9 probably acts to inhibit translation while creation of another hairpin loop which exposes the initiation codon is responsible for the increased expression.

The plasmid pNP14 was constructed, such that it had the S-D to AUG sequence of pNP9, but the coding sequence of pNP11. The expression plasmid pNP14, therefore, differed from pNP9 in only three residues, those in the degenerate position of three codons, yet strains containing pNP14 expressed over ninety times more μ than those containing pNP9. This increase in expression cannot be explained on the basis of the three residue changes optimizing codon usage, for they introduce less favoured codons, as judged by the frequency of their occurrence in strongly expressed *E. coli* genes.

The increased expression is best explained by the abolition of the RBS hairpin loop in pNP9. The magnitude of the increases in expression observed, and the few residue changes introduced, makes it difficult for these results to be interpreted in terms of changes in mRNA stability.

The expression of pNP8, which encodes the trpE-μ fusion protein from a native trpE RBS, was also examined, and found to express at approximately the same level as pNP14. This may indicate that the RBS of pNP14 is equivalent to that of trpE in terms of its efficiency in directing translation initiation. The native trpE RBS of pNP8 and that of pNP14 were found to have no secondary structures hindering the use of their RBS sequences.

Expression of μ Protein in *E. Coli*

E. Coli B cells containing the μ expression plasmid pNP11 were grown under inducing conditions and soluble and insoluble extracts prepared, and analysed by SDS-PAGE. A novel band was seen after staining the gel with Coomassie blue in the lane containing proteins from the insoluble fraction (see FIG. 6, lane 2). This band was not seen in the negative control lane which contained proteins from the same fraction from cells harbouring pCT70 (see FIG. 6, lane 3). The novel band was found to migrate to a position corresponding to a protein of a molecular weight within less than 5% of the actual molecular weight of non-glycosylated μ of 62.5 Kd. A duplicate set of lanes were transferred to nitrocellulose, and Western blotted. Alignment of the stained gel and the blot autoradiogram confirms that this novel band is antigenically related to IgM (see FIG. 6, lanes 4 and 8). No band was found in extracts from cells containing pCT70 (see FIG. 6, lanes 5 and 7). Only a low amount of μ was found in the soluble fraction (see FIG. 6, lane 6).

A greatly increased level of expression of μ was found in *E. Coli* B compared to HB101. Pulse chase analysis demonstrated that in *E. Coli* B, a similar level of μ protein was detected after a 60 minute chase (FIG. 7, lane 3) as was seen after the initial labelling period (FIG. 7, lane 1). In HB101, however, very little μ protein could be seen after a 10 minute chase (FIG. 7, lane 7), and none after 30 minutes (FIG. 7, lane 8), compared to the amount detected after the initial labelling period (FIG. 7, lane 5).

For pulse chase analysis, inductions were set up as described above, except that the medium used consisted of: proline (0.3 g/L), leucine (0.1 g/L), Difco methio-

nine assay medium (5 g/L), glucose (60 mg/L), thiamine (10 mg/L), $CaCl_2$ (22 mg/L), $MgSO_4$ (0.25 g/L)m and carbenicillin (0.1 g/L). During exponential growth cells were pulse labelled with 30 μCi/ml L-[^{35}S]methionine for 2 minutes, after which unlabelled methione (100 μg/ml) was added and the incubation continued for the times indicated.

Induced *E. Coli* B cells harbouring pNP14 when examined by phase contrast microscopy were found to contain inclusion bodies.

Expression of λ Light Chain in *E. Coli*

A fresh 1 ml overnight culture of *E. Coli* HB101 or RV308 containing the plasmid under study was grown in L Broth (Maniatis et al,, Molecular Cloning, Cold Spring Harbor Laboratory, 1982), supplemented with carbenicillin to 100 μg/ml.

A 1:100 dilution of this culture was made into M9 medium (Maniatis, 1982, op.cit.) supplemented with glucose, vitamin B_1, carbenicillin, leucine and proline and the cultures shaken at 37° for 5–6 hours. At this time ^{35}S-methionine was added for 5–20 minutes. The cells were then harvested by centrifugation, lysed by boiling in 1% SDS for 2 minutes and diluted by addition of a buffer consisting of 2% Triton X-100, 50mM Tris pH 8, 0.15M NaCl and 0.1 mM EDTA. Immunoprecipitations were carried out by addition of antisera to aliquots of labelled *E. Coli* extracts and, after incubation at 4° overnight, immune complexes were isolated by binding to Staphylococcus aureus fixed cells. The complexes were dissociated by boiling in 60 mM Tris pH 6.8 buffer containing 10% glycerol, 3% SDS and 5% mercaptoethanol and the liberated proteins analysed on 10% or 12.5% acrylamide/SDS gels (Laemmli, UK. Nature, 227, 680–685, 1970). Gels were stained with Coomassie Brilliant blue to visualise the protein bands while labelled proteins were detected on Fuji-RX film by fluorography using 1M sodium salicylate.

HB101 cells containing pNP3 and other plasmids were grown under inducing conditions to an OD_{600}=0.6 and the proteins present examined by specific immunoprecipitation substantially as described above. The results obtained are given in FIG. 8, in which lanes 1 and 2 respectively were extracts from pCT54-19 and pNP3, immunoprecipitated with normal rabbit serum; lanes 3, 4 and 5 were extracts immunoprecipitated with rabbit anti-lambda serum and represent pNP4 (lane 3), pNP3 (lane 4) and pCT54-19 (lane 5). The position of unlabelled lambda protein from MOPC104E is indicated on the left hand side of FIG. 8. All of the plasmids produced a protein which reacted with rabbit anti-mouse λ light chain serum and comigrated with authentic λ_1 light chain from the mouse myeloma MOPC104E. pNP3 however produced the most of this protein (compare lane 4 with lanes 3 and 5 in FIG. 8). No such band was detected with control immunoprecipitations using normal rabbit serum (FIG. 8, lanes 1 and 2). Studies using brief pulses with ^{35}S-methionine followed by chasing with excess cold methionine indicated that the recombinant λ light chain had a ½ life of about 20 minutes.

In vitro transcription/translation (Pratt et al, Nucleic Acids Research, 9, 4459–447, 1981) confirmed that pNP3 coded for a protein which comigrated with authentic λ light chain. This 25 Kd product was synthesized in vitro at a rate comparable to that of β-lactamase indicating that it is synthesised in vivo at a level of 0.5% of total *E. Coli* proteins. This figure is in good agreement with the percentage of recombinant λ product synthesised in vivo (0.4–2%) as determined from the following equation:

$$\% \text{ specific } CPM = \frac{100 \times (CPM \text{ with anti-}\lambda) - (CPM \text{ with normal serum})}{\text{total } CPM}$$

The procedure used to disrupt cells was as follows (Emtage J S et al, Proc. Natl. Acad. Sci., 80, 3671–3715, 1983). *E. Coli* HB101/pNP3 grown under inducing conditions were harvested and resuspended in 0.05M Tris pH 8, 0.233M NaCl, 5% glycerol containing 130 g/ml of lysozyme and incubated at 4° C. or room temperature for 20 minutes. Sodium deoxycholate was then added to a final concentration of 0.05% and 10 μg of DNAase 1 (from bovine pancreas) was added per g wet wt of *E. Coli.* The solution was incubated at 15° C. for 30 minutes by which time the viscosity of the solution had decreased markedly. The extract was centrifuged (at 10,000×g for 15 minutes for small volumes (1 ml) or 1 hour for larger volumes) to produce a soluble and an insoluble fraction.

For immunoprecipitations, the soluble fraction was diluted in the Triton-containing buffer described above and the insoluble fraction solubilised by boiling in 1% SDS followed by dilution in Triton-containing buffer. HB101 cells containing pNP3 or pNP4 were grown under inducing conditions, pulse labelled with [^{35}S]methionine, separated into soluble and insoluble fractions and analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The results obtained are shown in FIG. 9 in which lanes 1 and 3 correspond to the soluble fraction from HB101-pNP3 immunoprecipitated with normal rabbit serum and rabbit anti-lambda serum respectively; lanes 2 and 4 correspond to the insoluble fraction from HB101-pNP3 immunoprecipitated with normal rabbit serum and rabbit anti-lambda serum respectively, lanes 5 and 7 correspond to the soluble fraction from HB101-pNP4 immunoprecipitated with normal rabbit serum and rabbit anti-lambda serum respectively; lane 9 corresponds to the soluble fraction from HB101-pNP3, and lane 10 corresponds to the insoluble fraction from HB101-pNP3. The position of unlabelled lambda protein from MOPC104E is indicated on the left hand side of FIG. 9. When such a procedure was carried out using pNP3, the recombinant λ light chain protein was present in the insoluble fraction (FIG. 9, lane 4) rather than the soluble fraction (FIG. 9, lane 3). No 25 Kd band comigrating with authentic λ_1 light chain was present when either the soluble or insoluble fraction were immunoprecipitated using normal rabbit serum (FIG. 9, lanes 1 and 2). By inference it is likely that all Ig light chains will fractionate as in this specific example.

In the absence of specific immunoprecipitation a novel protein band was not visible from extracts of HB101 containing pNP3 nor was the λ protein found to accumulate. However, there was a dramatic difference when pNP3 was induced in the K12 strain E103s. In this strain λ protein was found to accumulate during induction until the cells reaches stationary phase (FIG. 10, lanes 3–5) to a level of about 150 times that found in HB101 as determined by an ELISA (enzyme linked immunosorbent assay). These cells were found to contain inclusion bodies which appeared refractile under light microscopy, a phenomenon characteristic of high

level expression of foreign proteins. An estimate of the pecentage of total *E. coli* protein represented by recombinant λ protein was obtained by separating the proteins by gel electrophoresis, staining them, with Coomassie blue and scanning the stained gel with a Joyce-Loebl chromoscan 3. This method showed that λ was the major protein present (FIG. 10, lane 5) and represented 13% or total *E. coli* protein. The λ protein had a half-life of 20 minute in HB101 but accumulated to very high levels in E103s, suggesting that lambda protein was much more stable in the latter strain. After cell lysis and centrifugation of HB101 or E103s containing pNP3, λ light chain was detected in the insoluble (FIG. 10, lanes 7 and 10) but not in the soluble fractions (FIG. 10, lanes 6), as determined by Coomassie blue staining. The identity of the major Coomassie blue stained band as λ protein was confirmed by Western blot analysis (FIG. 10, lanes 8–10). The presence of such immunoreactive bands was specific to pNP3 containing cells. When extracts from cells containing pCT70, a prochymosin expressing plasmid, were subjected to the same analysis no bands were detected. This more sensitive technique showed that a small amount of the λ protein was in the soluble fraction (FIG. 10, lane 9). The presence of a number of distinct immunoreactive proteins all smaller than full-length protein were also detected. These may result from proteolytic degradation of of λ protein, from premature termination of transcription or from internal initiation of translation.

Expression of μ and Lambda Polypeptides in the same Bacterial Cell

Each of the Ig μ and λ genes in expression plasmids were transformed into the same *E. Coli* cell to direct the synthesis of both Ig μ and λ polypeptides. In order to overcome plasmid incompatibility and provide a second antibiotic resistance marker, the trp promoter and λ sequences were excised from pNP3 on a Hind III-Bam HI fragment and inserted into the Hind III-Bam HI fragment of pACYC 184. The resultant plasmid pACYC λ caused the *E. coli* to grow very poorly. This weak growth was thought to result from read-through of RNA polymerase into the origin of replication. However, the inhibition of growth was virtually eliminated by the cloning in of the baceteriophage T7 transcriptional terminator at the Hind III site of plasmid pACYC λ.

This terminator functions in both orientations. The resultant plasmid pAC λ T7-1 has a chloroamphenicol resistance gene and an origin compatible with the pBR 322-derived origin on pNP14, the Ig μ expressing plasmid. Transformation of both plasmids in the same *E. Coli B* was achieved in two steps. Firstly pNP14 was introduced followed by pAC λ T7- I in two sequential transformations to give ampicillin and chloramphenicol resistant clones.

E. Coli B cells derived form this double-transformant clone showed the presence of inclusion bodies and two novel polypeptide bands on stained gels of the insoluble fraction after lysis. These two bands correspond both with immunological activity by Western blotting for Ig μ and Ig λ and in their expected molecular weights. It has thus been shown that the double-transformant clone expresses both the heterologous genes. This had not hitherto been shown.

The presence of λ light chain in the insoluble fraction was a useful purification step since it both concentrated the protein and separated if from the bulk of *E. Coli* soluble proteins.

For further purification of the λ light chain, the cell debris were dissolved in 10 mM Tris-HCl pH8.0, 25% formamide, 7M urea, 1 mM EDTA and 2 mM dithiothreitol. This material was loaded onto a DEAE Sephacel column (Pharmacia) (1×25 cm at a flow rate of 5 ml/hr) which had been equilibrated in 9M urea, 10 mM Tris-HCl pH 8.0, 1 mM EDTA and 2 mM DTT. The DEAE Sephacel column was developed using a 0–150 mM NaCl gradient in loading buffer. The eluted peak λ light chain immunoreactivity, corresponding to the major peak of protein, was diluted to a final concentration of 2.25M urea, 10 mM Tris-Hcl pH 8.0, 1 mM EDTA, 2 mM DTT and loaded onto an octylSepharose column (Pharmacia) (2.5×10 cm). Material was eluted by use of a urea gradient of 2.25–9M urea. The peak material was pooled, dialysed into ammonium bicarbonate and lyophilised. Following this step, only a single band of Coomassie blue stainable material corresponding to recombinant λ protein was visualised by SDS-PAGE.

The μ heavy chain was purified from 9M urea solubilised pellets by anion exchange chromatogrpahy and chromatofocussing (Pharmacia).

It was of great interest to determine whether the concomitant expression of μ and λ would lead to the formation of functional IgM. In order to determine this, extracts were made from *E. coli* containing both Ig μ and λ polypeptides and these tested for antigen binding. We used a two-site sandwich ELISA which detects μ chain binding to haptenalated bovine serum albumin (NIP-caproate-BSA).

In the NIP binding assay, bovine serum albumin (BSA) is reacted with an equimolar amount of NIP-Cap-N-hydroxy-succinamide at pH 7.5 in 10 mM phosphate buffer. The resulting NIP-Cap-BSA is separated from free NIP-Cap on a G-50 Sephadex column.

Microtitre plates (96 well Nunc Immuno Plate 1) are coated with 100 μl of a solution of 10 μg/ml NIP-BSA in sodium carbonate/sodium bicarbonate 0.1M, pH 9.6 buffer (coating buffer) overnight at 4° C.

The coated plates are then blocked for non-specific binding by addition of 100 μl of 0.5% casein in coating buffer (blocking buffer) and incubating at 37° C. for 1 hour.

The coated and blocked plates are then washed three times in 150 mM NaCl, 0.05% NP40, 20 mM Tris-HCl, pH 7.8 buffer (washing buffer).

The washed plates are shaken free of excess washing buffer and samples are added in their buffer or typically in 0.5% casein in washing buffer (sample buffer) to a 100 μl final volume.

In order to demonstrate the nature of the binding to NIP-BSA, samples for testing were supplemented with either NIP or NP is free solution at various concentrations (from 60 to 0.3 μM for NIP or from 600 to 3 μM for NP).

The plates with samples are then incubated at 37° C. for one hour and thereafter washed three times with washing buffer.

The washed plates are then innoculated with 100 μl of anti μ-peroxidase conjugate (1:1000 dilution; TAGO Inc) in sample buffer, and incubated for one hour at 37° C. The plates are then washed three times in washing buffer.

The washed plates are innoculated with 100 μl of 0.1M Na acetate-citrate, pH 6.0, 0.1 mg/ml tetramethyl-

benzidine, 13 mM H_2O_2 (peroxidase substrate) and incubated at room temperature for one hour. The reaction is terminated by the addition of 25 µl of 2.5M H_2SO_4.

The microtitre plates are then read in a plate reader (Dynatech) at 450 λm with a reference of 630 λm. The A_{450} was related to the level of a standard protein, Bl-8 anti-NIP IgM.

This assay demonstrates sensitivity to 60 pg of Bl-8 IgM. The extracts were prepared as soluble and insoluble material. The insoluble material was solubilized in the same buffer used in lysis but containing 8M urea followed by its dilution for assay.

In order to obtain activity for the Ig µ and λ, extracts were made of the insoluble fraction and these dialysed into buffer conditions in which disulphide interchange will occur at a higher frequency.

Production of functional antibodies from *E. Coli* expressing both heavy and light chains was achieved by lysing the cells and clarifying the supernatant by centrifugation. The insoluble material was washed, followed by sonication (3 times for 3 minutes), and finally dissolved in 9M urea, 50 mM glycine-NaOH pH 10.8, 1 mM EDTA, and 20 mM 2-mercaptoethanol. This extract was dialysed for 40 hours against 3 changes of 20 vols. of 100 mM KCl, 50 mM glycine-NaOH pH 10.8, 5% glycerol, 0.05 mM EDTA, 0.5 mM reduced glutathione and 0.1 mM oxidised glutathione. The dialysate was cleared by centrifugation at 30,000 g for 15 minutes and loaded directly onto DEAE Sephacel, followed by development with a 0–0.5M KCl linear gradient in 10 mM Tris-HCl, 0.5 mM EDTA, pH 8.0.

The purified Ig µ and λ were treated as above, except that no anion exchange chromatography was carried out. The preparation was finally dialysed into phosphate buffered saline, 5% glycerol, 0.01% sodium azide and 0.5 mM EDTA pH 7.4.

The results from assays of material processed in this way indicated that some activity was obtained. The level of activity obtained in this way was too low to do any detailed studies on, so the resultant dialysate was purified by anion exchange chromatography (FIG. 11). This process resulted in the isolation of significant NIP-cap-BSA binding activity over that of background binding to BSA (FIG. 11). The assay of the fractions for the level of Ig µ, expressed as Bl-8 IgM equivalents demonstrated two peaks of activity. This was not found to correlate with full length Ig µ by Western blotting. The first peak observed may represent a fragment of Ig µ. The separation of NIP-CAP-BSA binding activity from the majority of full length Ig µ and protein indicates that the hapten binding activity is contained within a particular molecular species formed at low efficiency.

The processing of insoluble material obtained from Ig µ expression in *E. Coli* produced a similar IgM protein profile but without NIP-cap-BSA binding activity. This demonstrates that the activity recovered was a property of the combined immunoglobulin expression, not of some *E. Coli* factor, or of the Ig µ heavy chain alone.

Further studies of the characteristics of the hapten (NIP-cap-BSA) binding were carried out. As samples were diluted they showed less binding to hapten in a very similar way to the original antibody (FIG. 12). Free hapten was found to inhibit most of the binding activity in both indiluted and diluted samples. Using Bl-8 antibody as a standard for both IgM and hapten binding, the specific activity of the assembled antibody was calculated to be 1.4×10^4 gm/gm of IgM equivalents. This value demonstrates the inefficient recovery of activity, but possibly represents an underestimate of the specific activity due to an overestimate of full-length Ig µ in these fractions, as described above.

Heteroclitic Nature of Recombined Antibody

Detailed specificity of binding to NIP-cap-BSA was investigated by comparing the assembled antibodies with Bl-8 IgM in the presence of free NIP-cap and NP-cap (FIG. 13). Both Bl-8 IgM and the assembled antibodies showed that higher NP-cap than NIP-cap concentrations were required to inhibit NIP-cap-BSA binding.

The heteroclitic nature is demonstrated by the molar ratio of NIP to NP at 50% inhibition. The concentrations of NIP and NP at 50% inhibition (I50) were found to be similar for both Bl-8 and the assembled antibodies as shown in Table 2. Also the NP I50/NIP I50 ratios were similar (Table 2).

TABLE 2

Hapten concentration at 50% inhibition (I50) of binding of antibodies to NIP-cap-BSA solid phase. SD = Deviation.

	NIP I50 uM	NP I50 uM	NP I50/ NIP I50
Bl-8 IgM	0.13 (SD, 0.05)	3.7 (SD, 2.9)	29
Fraction 26	0.24 (SD, 0.09)	1.9 (SD, 0.4)	6
Fraction 27 and 28	0.11 (SD, 0.02)	1.1 (SD, 0.3)	10
Purified µ and λ	0.4	0.84	22

It has thus been shown that not only is it possible to express µ and λ proteins having intact variable domains, but also that it is possible to express both µ and λ proteins on compatible plasmids in the same cell. This latter has not hitherto been disclosed or even suggested. Moreover, it proved possible to derive functional Ig molecules from *E. Coli* cells which expressed both µ and λ proteins.

It has thus been shown that the process of the present invention produces a functional Ig molecule by recombinant DNA techniques.

Construction of Expression Plasmids for Yeast

The plasmids used for expression in yeast (*Saccharomyces cerevisiae*) are based on pBR322 and the yeast 2 micron plasmid. These plasmids are the subject of copending European Patent Application EP 0 073 635, the disclosure of which is incorporated hereby reference. The yeast phosphoglycerate kinase gene (PGK) provides the 5' sequence necessary for initiation of transcription and insertion of genes can be made at the unique BgIII site in the plasmid pMA 3013 described in the above mentioned European patent application. This plasmid has now been renamed pMA91 as is so referred to hereinafter.

(a) met-mu (µ)

BglII and BclI produce compatible 5'-GATC ends. The met-µ gene from pNP2 was excised on a partial BclI fragment and ligated into the unique BglII site of pMA91.

(b) Pre-mu (µ)

The plasmid pCT54 u containing the full lengths pre-µ cDNA was digested with Hind III. This cuts at the 3' side of the µ cDNA. This Hind III site was changed to a BclI site by incubation with T_4 DNA polymerase in the presence of all 4 nucleotides. The linker R107 of sequence TTTTGATCAAAA, which contains an internal BclI was ligated to the DNA obtained above. After ligation one aliquot of the resultant

DFNA was digested with BcII and AccI and the μ AccI-BcII fragment was isolated from the resultant mixture by gel electrophoresis. A separate aliquot of the resultant DNA was digested with MboII and ligated with chemically synthesised oligonucleotide linkers R121 and R112 which have cohesive BcII and MboII ends.

R121 5' GATCAATGGGATGGAGCTGT 3'

R112 5' TTACCCTACCTCGAC 3'

The ligation reaction was terminated and the resultant DNA digested with AccI to give a μ MboII-AccI fragment which was isolated from the resultant mixture by gel electrophoresis on a 5% polyacrylamide gel.

The two μ fragments obtained above were ligated together and then digested with BcII to eliminate unwanted ligation products after which the resultant pre-μ DNA was ligated to BgIII cut plasmid pMA91.

The resultant ligation mix was used to transform *E. Coli* strain HB101 to ampicillin resistance. A colony of the transformed bacteria was isolated and was found to contain a plasmid exhibiting the predicted enzyme digestion pattern. The 5' end of the inserted DNA from this plasmid was sequenced and shown to have the anticipated sequence for a pre-μ cDNA.

(c) met-lambda (λ)

The met-λ DNA was cloned into the BgIII site of plasmid pMA91. Plasmid pCT54 Clone 1 was cut with Hind III to the 3' side of the met-λ cDNA and this cleavage site was altered to a BclI site in a similar manner as previously described for pNP2. Following this the plasmid DNA was cut 5' of the λ gene at the BclI site and the resultant λ DNA was cloned into the BglII site of pMA91.

(d) Pre-lambda (λ)

Pre-λ cDNA was reconstructed into pMA 91 as follows. The plasmid pCT54 was digested with BclI and Hind III and the resultant vector DNA was isolated by gel electrophoresis and ligated together with two synthetic oligonucleotides, R162 and R163, and the two fragments from plasmid pAT λ 1-15.

R162 5' GATCAATGGCCTGGATT 3'

R163 5' GTGAAATCCAGGCCATT 3'

These fragments were produced by digesting pAT λ 1-15 with FokI and Hind III and isolating the 5'300 base pair FokI fragment and the 3' 600 base pair FokI-Hind III fragment by gel electrophoresis. The resultant ligation mixture was used to transform *E. Coli* strain HB101 to ampicillin resistance and a colony of bacteria containing a plasmid having the predicted correct restriction enzyme digestion pattern was isolated. The 5' end of the inserted DNA was sequenced and found to have the anticipated sequence of a pre-λ cDNA. This plasmid was digested with Hind III and the resultant Hind III cleaved ends converted to a BclI site by blunting with T_4 polymerase and ligating with the linker R107. The resultant plasmid was then digested with BclI and the pre-λ cDNA isolated on a BclI fragment by gel electrophoresis. This BclI fragment was then ligated to BglI cut pMA91 to give a pMA 91 plasmid containing the full length pre-λ cDNA.

Expression of Immunoglobulin Genes in Yeast

The pMA91 derivative plasmids containing the pre-λ and pre-μ genes as prepared above were used to transform *Saccharomyces cerevisiae* yeast host organisms and the pre-λ and pre-μ genes were expressed by the transformed cells.

Saccharomyces cerevisiae strain MD46 when transformed with pMA91 pre-λ or pMA91 pre-μ gave rise to colonies which expressed immunoreactive proteins as revealed on Western blots. Yeast cells containing plasmid pMA91 pre-λ produced a protein which reacted with anti-λ antiserum and co-migrated on polyacrylamide gels with bacterially synthesised mature λ. Similarly yeast cells containing pMA 91 pre-μ produced a protein which reacted with anti-μ antiserum and co-migrated on polyacrylamide gels with bacterially synthesised mature λ protein. These observations indicate that both pre-μ and pre-μ are being processed to the corresponding mature proteins within the yeast host cell environment.

Additionally the λ protein product (but not the λ protein) was shown to be glycosylated. Cells were incubated in the presence or absence of tunicamycin at a concentration of 15 μg/ml. This compound specifically arrests the N-linked glycosylation of proteins. Cell extracts derived from cells incubated in the absence of tunicamycin showed higher molecular weight bands (as revealed by Western blotting) whilst extracts from cells incubated in the presence of tunicamycin showed no such higher molecular weight bands.

When cells were lysed with glass beads and the soluble and insoluble fractions examined it was found that the μ and λ proteins were exclusively in the insoluble fractions, as determined by Western blotting.

Secretion

After incubation transformed yeast cells were spun down and 1 ml volumes of supernatant were removed and passed through BSA-coated 0.2 μM Millipore filters to remove any remaining cellular material. ELISA assays for μ and λ protein were then carried out on the filtered supernatants. Only supernatants from pMA91 pre-λ harbouring cells showed detectable levels of immunoglobulin protein. Increased amounts of λ protein was detected in the supernatant when cells were grown in minimal medium to OD_{660}=0.2 and then spun down and resuspended in YPAB and harvested at OD_{660}=1-.0-1.5. Thus the λ protein but not the μ protein is secreted from yeast cells.

Intracellular Location

After incubation cells containing pMA91 pre-μ were converted to spheroplasts and fixed in 5% acetic acid/95% ethanol (v/v) and incubated with fluorescein conjugated goat anti-mouse μ. The fixed cells were examined by fluorescent microscopy and the μ protein was found to be localised in the periphery of the spheroplasts and especially in vacuoles.

Expression of both μ and λ Proteins in the Same Cells

In order to express both genes in the same host cells it was necessary to provide compatible plasmids for use in transformation. The pre-λ gene was excised from pMA91 pre-λ on a Hind III fragment and inserted into the Hind III site of plasmid pLG89 (Griti L, and Davies J, Gene, 25 179-188, 1983). This plasmid contains a ura3 marker which can be used as a positive selection for

transformed host cells. A convenient ura3 host organism is *S. cerevisiae* strain X4003-5B. Both plasmids, pMA91 pre-μ and pLG89 pre-λ, were transformed into this strain, and colonies were grown which contained together both the ura3 and leu2 markers. After incubation of the transformed cells both λ and μ proteins together were detected in the same cultures of X4003-5B using ELISA techniques. Levels of expression were comparable to those obtained for the individual genes in MD46 cells and for the λ gene alone in X4000-5B.

In addition, to check for assembly of the λ and μ proteins in vivo the following procedure was followd.

After the growth to an OD_{660}≃1, the transformed X4003-5B cell were spun down and resuspended in buffer, either 5 mM borate buffer at pH 8.0 or phosphate buffer at pH 5.8 either with or without detergent (e.g. 0.5% Triton X). The suspended cells were then lysed by vortexing with glass heads in buffer as above and the insoluble material was spun down. After centrifugation aliquots of the supernatant were assayed in a NIP binding assay (as described previously for assembled *E. Coli* μ and λ proteins). Specific antigen binding activity was detected in the supernatant and this activity was shown to be specifically competed out by free NIP (the specific antigen). These results indicated that μ and λ protein were expressed within the X4003-5 B cells and assembled into functional immunoglobulin molecules in vivo.

In the above experiments the culture medium used for incubating the transformed cells was yeast minimal medium (containing, per liter, 6.7 g DIFCO yeast nitrogen base without amino acids, 10 g glucose, and 200 mg each of histidine, tryptophan, methionine and adenine). When the λ protein alone was being expressed the medium contained in addition 200 mg/l of leucine, and when the μ protein alone was being expressed the medium contained in addition 200 mg/l of uracil.

We claim:

1. A process for producing an Ig molecule or an immunologially functional Ig fragment comprising at least the variable domains of the Ig heavy and light chains, in a single host cell, comprising the steps of:

(i) transforming said single host cell with a first DNA sequence encoding at least the variable domain of the Ig heavy chain and a second DNA sequence encoding at least the variable domain of the Ig light chain, and

(ii) independently expessing said first DNA sequence and said second DNA sequence so that said Ig heavy and light chains are produced as separate molecules in said transformed single host cell.

2. The process according to claim 1 wherein said first and second DNA sequences are present in different vectors.

3. The process according to claim 1 wherein said first and second DNA sequences are present in a single vector.

4. A process according to claim 2 wherein the or each vector is a plasmid.

5. A process according to claim 4, wherein the plasmid is col E1, pcR1, pBR322, RP4 or phage DNA.

6. A process according to claim 1 wherein the host cell is a baceterium or a yeast.

7. A process according to claim 6, wherein the host cell is *E. Coli, B. subtilis* or *S. cerevisiae.*

8. A process according to claim 7, wherein the host cell is *E. Coli* strain HB101, X1776, X2887, PS410, MRC 1 RV308, B or E1035.

9. A process according to claim 1 wherein the Ig heavy and light chains are expressed in the host cell and secreted therefrom as an immunologically functional Ig molecule or Ig fragment.

10. A process according to claim 1 wherein the Ig heavy and light chains are produced in insoluble or membrane bound form and are solubilised and allowed to refold in solution to form an immunologically functional Ig molecule or Ig fragment.

11. A process according to claim 1, wherein the DNA sequences code for the complete Ig heavy and light chains.

12. A process according to claim 1, wherein said first or said second DNA sequence further encodes at least one constant domain, wherein the constant domain is derived from the same source as the variable domain to which it is attached.

13. A process according to claim 1, wherein said first or said second DNA sequence further encodes at least one constant domain, wherein the constant domain is derived from a source different from that from which the variable domain to which it is attached is derived.

14. A process according to claim 1, wherein said first and second DNA sequences are derived from one or more monoclonal antibody producing hybridomas.

15. A vector comprising a first DNA sequence encoding at last a variable domain of an Ig heavy chain and a second DNA sequence encoding at least a variable domain of an Ig light chain wherein said first DNA sequence and said second DNA sequence are located in said vector at different insertion sites.

16. A vector according to claim 15, which is a plasmid.

17. A host cell transformed with a vector according to claims 15.

18. A transformed host cell comprising at least two vectors, at least one of said vectors comprising a DNA sequence encoding at least a variable domain of an Ig heavy chain and at least another one of said vectors comprising a DNA sequence encoding at least the variable domain of an Ig light chain.

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United States Patent [19]

Cabilly et al.

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[54] **RECOMBINANT IMMUNOGLOBIN PREPARATIONS**

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Primary Examiner—Jayme Huleatt

(List continued on next page.)

[57] **ABSTRACT**

Altered and native immunoglobulins, including constant-variable region chimeras, are prepared in recombinant cell culture. The immunoglobulins contain variable regions which are immunologically capable of binding predetermined antigens. Methods are provided for refolding directly expressed immunoglobulins into immunologically active form.

7 Claims, 15 Drawing Sheets

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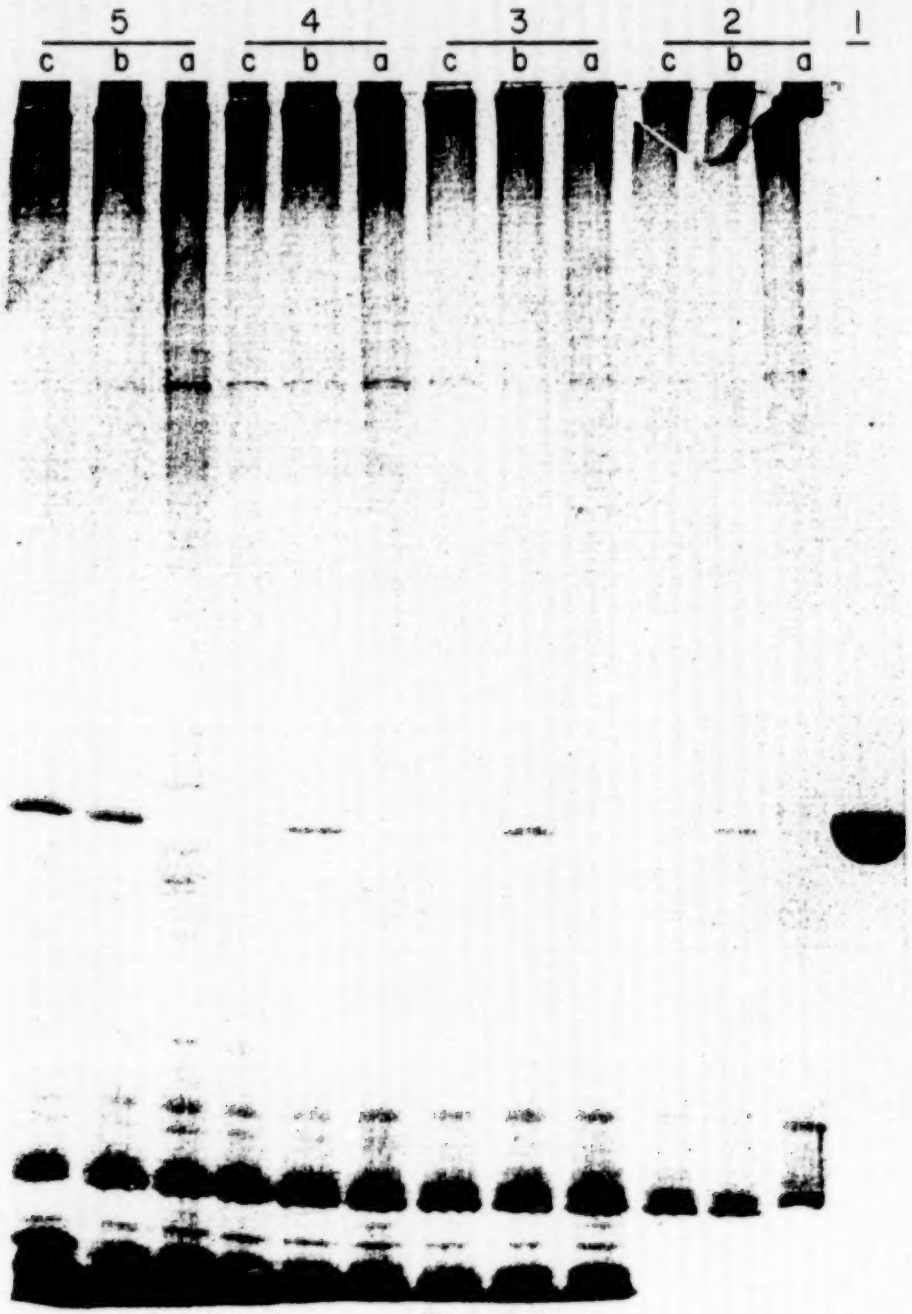

Fig. 8A.

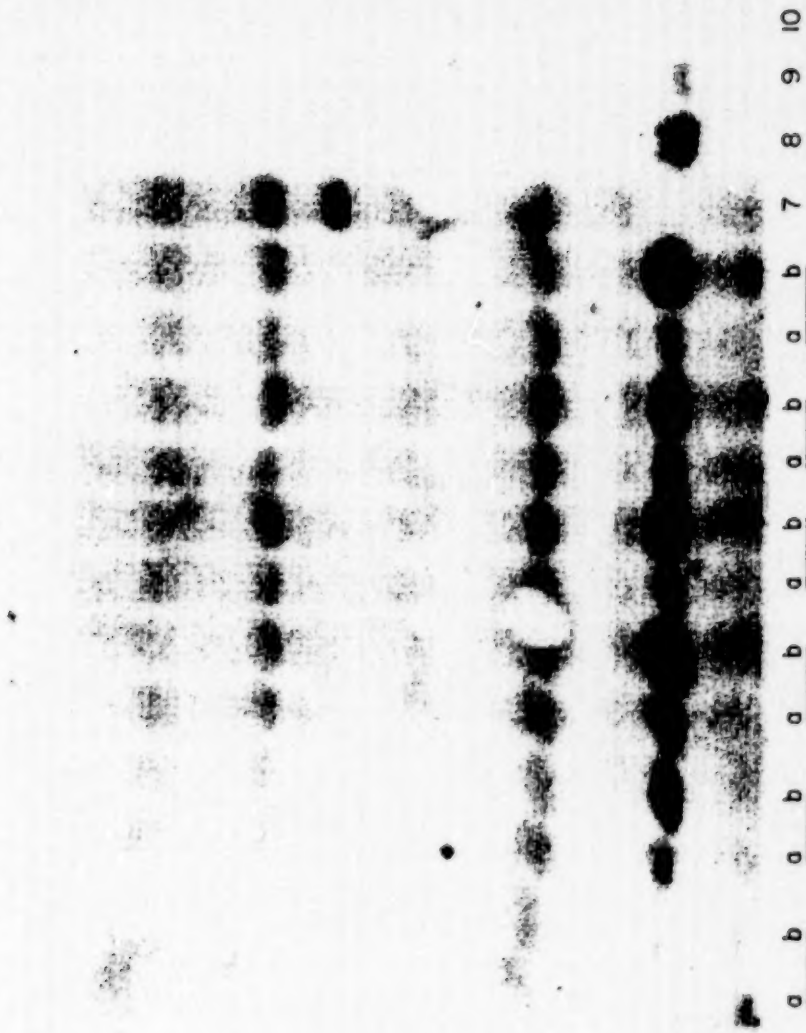

Fig. 8B.

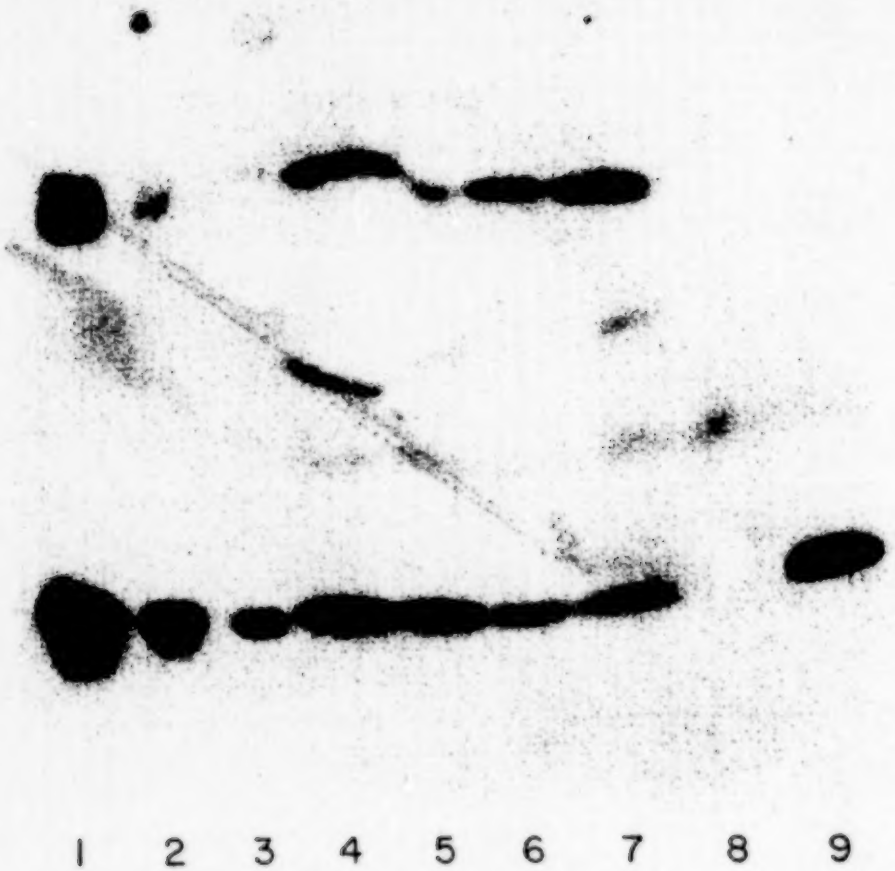

Fig. 8C.

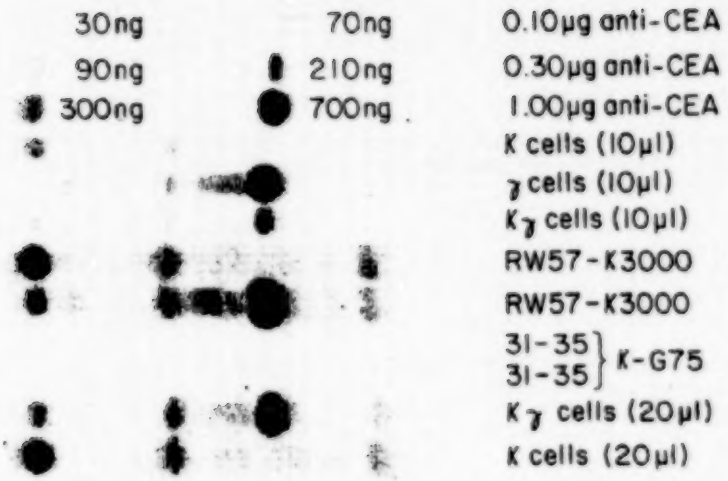
30ng
70ng
0.10μg anti-CEA
90ng
210ng
0.30μg anti-CEA
300ng
700ng
1.00μg anti-CEA
K cells (10μl)
γ cells (10μl)
Kγ cells (10μl)
RW57-K3000
RW57-K3000
31-35
31-35
K-G75
Kγ cells (20μl)
K cells (20μl)

Fig. 9.

RECOMBINANT IMMUNOGLOBIN PREPARATIONS

BACKGROUND OF THE INVENTION

This invention relates to the field of immunoglobulin production and to modification of naturally occuring immunoglobulin amino acid sequences. Specifically, the invention relates to using recombinant techniques to produce both immunoglobulins which are analogous to those normally found in vertebrate systems and to take advantage of these gene modification techniques to construct chimeric or other modified forms.

A. Immunoglobulins and Antibodies

Antibodies are specific immunoglobulin polypeptides produced by the vertebrate immune system in response to challenge by foreign proteins glycoproteins, cells, or other antigenic foreign substances. The sequence of events which permits the organism to overcome invasion by foreign cells or to rid the system of foreign substances is at least partially understood. An important part of this process is the manufacture of antibodies which bind specifically to a particular foreign substance. The binding specificity of such polypeptides to a particular antigen is highly refined, and the multitude of specificities capable of being generated by the individual vertebrate is remarkable in its complexity and variability. Thousands of antigens are capable of eliciting responses, each almost exclusively directed to the particular antigen which elicted it.

Immunoglobulins include both antibodies, as above described, and analogous protein substances which lack antigen specificity. The latter are produced at low levels by the lymph system and in increased levels by myelomas.

A.1 Source and Utility

Two major sources of vertebrate antibodies are presently utilized—generation in situ by the mammalian B lymphocytes and in cell culture by B-cell hybrids. Antibodies are made in situ as a result of the differentiation of immature B lymphocytes into plasma cells, which occurs in response to stimulation by specific antigens. In the undifferentiated B cell, the portions of DNA coding for the various regions on the immunoglobulin chains are separated in the genomic DNA. The sequences are reassembled sequentially prior to transcription. A review of this process has been given by Gough, *Trends in Biochem Sci.* 6: 203 (1981). The resulting rearranged genome is capable of expression in the mature B lymphocyte to produce the desired antibody. Even when only a single antigen is introduced into the sphere of the immune system for a particular mammal, however, a uniform population of antibodies does not result. The in situ immune response to any particular antigen is defined by the mosaic of responses to the various determinants which are present on the antigen. Each subset of homologous antibody is contributed by a single population of B cells—hence in situ generation of antibodies is "polyclonal".

This limited but inherent heterogeneity has been overcome in numerous particular cases by use of hybridoma technology to create "monoclonal" antibodies (Kohler, et al., *Eur. J. Immunol.*, 6: 511 (1976)). In this process, splenocytes or lymphocytes from a mammal which has been injected with antigen are fused with a tumor cell line, thus producing hybrid cells or "hybridomas" which are both immortal and capable of producing the genetically coded antibody of the B cell. The hybrids thus formed are segregated into single genetic strains by selection, dilution, and regrowth, and each strain thus represents a single genetic line. They therefore produce immunoreactive antibodies against a desired antigen which are assured to be homogenous, and which antibodies, referencing their pure genetic parentage, are called "monoclonal". Hybridoma technology has to this time been focused largely on the fusion of murine lines, but human-human hybridomas (Olsson, L. et al., *Proc. Natl. Acad. Sci. (USA)*, 77: 5429 (1980)); human-murine hybridomas (Schlom, J., et al. (ibid) 77: 6841 (1980)) and several other xenogenic hybrid combinations have been prepared as well. Alternatively, primary, antibody producing, B cells have been immortalized in vitro by transformation with viral DNA.

Polyclonal, or, much more preferably, monoclonal, antibodies have a variety of useful properties similar to those of the present invention. For example, they can be used as specific immunoprecipitating reagents to detect the presence of the antigen which elicited the initial processing of the B cell genome by coupling this antigen-antibody reaction with suitable detection techniques such as labeling with radioisotopes or with enzymes capable of assay (RIA, EMIT, and ELISA). Antibodies are thus the foundation of immuno diagnostic tests for many antigenic substances. In another important use, antibodies can be directly injected into subjects suffering from an attack by a substance or organism containing the antigen in question to combat this attack. This process is currently in its experimental stages, but its potential is clearly seen. Third, whole body diagnosis and treatment is made possible because injected antibodies are directed to specific target disease tissues, and thus can be used either to determine the presence of the disease by carrying with them a suitable label, or to attack the diseased tissue by carrying a suitable drug.

Monoclonal antibodies produced by hybridomas, while theoretically effective as suggested above and clearly preferable to polyclonal antibodies because of their specificity, suffer from certain disadvantages. First, they tend to be contaminated with other proteins and cellular materials of hybridoma, (and, therefore, mammalian) origin. Second, hybridoma lines producing monoclonal antibodies tend to be unstable and may alter the structure of antibody produced or stop producing antibody altogether (Kohler, G., et al., *Proc. Antl. Acad. Sci (USA)* 77: 2197 (1980); Morrison, S. L., *J. Immunol.* 123: 793 (1979)). The cell line genome appears to alter itself in response to stimuli whose nature is not currently known, and this alteration may result in production of incorrect sequences. Third, both hybridoma and B cells inevitably produce certain antibodies in glycosylated form (Melchers, F., *Biochemistry*, 10: 653 (1971)) which, under some circumstances, may be undesirable. Fourth, production of both monoclonal and polyclonal antibodies is relatively expensive. Fifth, and perhaps most important, production by current techniques (either by hybridoma or by B cell response) does not permit manipulation of the genome so as to produce antibodies with more effective design components than those normally elicited in response to antigens from the mature B cell in situ. The antibodies of the present invention do not suffer from the foregoing drawbacks, and, furthermore, offer the opportunity to provide molecules of superior design.

Even those immunoglobulins which lack the specificity of atibodies are useful, although over a smaller spectrum of potential uses than the antibodies themselves. In presently understood applications, such immunoglobulins are helpful in protein replacement therapy for globulin related anemia. In this context, an inability to bind to antigen is in fact helpful, as the therapeutic value of these proteins would be impaired by such functionality. At present, such non-specific antibodies are derivable in quantity only from myeloma cell cultures suitably induced. The present invention offers an alternative, more economical source. It also offers the opportunity of cancelling out specificity by manipulating the four chains of the tetramer separately.

A.2 General Structure Characteristics

The basic immunoglobin structural unit in vertebrate systems is now well understood (Edelman, G. M., *Ann. N.Y. Acad. Sci.*, 190: 5 (1971)). The units are composed to two identical light polypeptide chains of molecular weight approximately 23,000 daltons, and two identical heavy chains of molecular weight 53,000-70,000. The four chains are joined by disulfide bonds in a "Y" configuration wherein the light chains bracket the heavy chains starting at the mouth of the Y and continuing through the divergent region as shown in FIG. 1. The "branch" portion, as there indicated, is designated the Fab region. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, with some subclasses among them, and the nature of this chain, as it has a long constant region, determines the "class" of the antibody as IgG, IgM, IgA, IgD, or IgE. Light chains are classified as either kappa or lambda. Each heavy chain class can be prepared with either kappa or lambda light chain. The light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages when the immunoglobulins are generated either by hybridomas or by B cells. However, if non-covalent association of the chains can be effected in the correct geometry, the aggregate will still be capable of reaction with antigen, or of utility as a protein supplement as a non-specific immunoglobulin.

The amino acid sequence runs from the N-terminal end at the top of the Y to the C-terminal end at the bottom of each chain. At the N-terminal end is a variable region which is specific for the antigen which elicited it, and is approximately 100 amino acids in length, there being slight variations between light and heavy chain and from antibody to antibody. The variable region is linked in each chain to a constant region which extends the remaining length of the chain. Linkage is seen, at the genomic level, as occuring through a linking sequence known currently as the "J" region in the light chain gene, which encodes about 12 amino acids, and as a combination of "D" region and "J" region in the heavy chain gene, which together encode approximately 25 amino acids.

The remaining portions of the chain are referred to as constant regions and within a particular class do not to vary with the specificity of the antibody (i.e., the antigen eliciting it).

As stated above, there are five known major classes of constant regions which determine the class of the immunoglobulin molecule (IgG, IgM, IgA, IgD, and IgE corresponding to γ, μ, α, δ, and ϵ heavy chain constant regions). The constant region or class determines subsequent effector function of the antibody, including activation of complement (Kabat, E. A., *Structural Concepts in Immunology and Immunochemistry*, 2nd Ed., p. 413-436, Holt, Rinehart, Winston (1976)), and other cellular responses (Andrews, D. W., et al., *Clinical Immunobiology*, pp 1-18, W. B. Sanders (1980); Kohl, S., et al., *Immunology*, 48: 187 (1983)); while the variable region determines the antigen with which it will react.

B. Recombinant DNA Technology

Recombinant DNA technology has reach sufficient sophistication that it includes a repertoire of techniques for cloning and expression of gene sequences. Various DNA sequences can be recombined with some facility, creating new DNA entities capable of producing heterologous protein product in transformed microbes and cell cultures. The general means and methods for the in vitro ligation of various blunt ended or "sticky" ended fragments of DNA, for producing expression vectors, and for transforming organisms are now in hand.

DNA recombination of the essential elements (i.e., an origin of replication, one or more phenotypic selection characteristics, expression control sequence, heterologous gene insert and remainder vector) generally is performed outside the host cell. The resulting recombinant replicable expression vector, or plasmid, is introduced into cells by transformation and large quantities of the recombinant vehicle is obtained by growing the transformant. Where the gene is properly inserted with reference to portions which govern the transcription and translation of the encoded DNA message, the resulting expression vector is useful to produce the polypeptide sequence for which the inserted gene codes, a process referred to as "expression." The resulting product may be obtained by lysis, if necessary, of the host cell and recovery of the product by appropriate purifications from other proteins.

In practice, the use of recombinant DNA technology can express entirely heterologous polypeptides—so-called direct expression—or alternatively may express a heterologous polypeptide fused to a portion of the amino acid sequence of a homologous polypeptide. In the latter cases, the intended bioactive product is sometimes rendered bioinactive within the fused, homologous/heterologous polypeptide until it is cleaved in an extracellular environment.

The art of maintaining cell or tissue cultures as well as microbial systems for studying genetics and cell physiology is well established. Means and methods are available for maintaining permanent cell lines, prepared by successive serial transfers from isolated cells. For use in research, such cell lines are maintained on a solid support in liquid medium, or by growth in suspension containing support nutriments. Scale-up for large preparations seems to pose only mechanical problems.

SUMMARY OF THE INVENTION

The invention relates to antibodies and to non-specific immunoglobulins (NSIs) formed by recombinant techniques using suitable host cell cultures. These antibodies and NSIs can be readily prepared in pure "monoclonal" form. They can be manipulated at the genomic level to produce chimeras of variants which draw their homology from species which differ from each other. They can also be manipulated at the protein level, since all four chains do not need to be produced by the same cell. Thus, there are a number of "types" of immunoglobulins encompassed by the invention.

First, immunoglobulins, particularly antibodies, are produced using recombinant techniques which mimic

the amino acid sequence of naturally occuring antibodies produced by either mammalian B cells in situ, or by B cells fused with suitable immortalizing tumor lines, i.e., hybridomas. Second, the methods of this invention produced, and the invention is directed to, immunoglobulins which comprise polypeptides not hitherto found associated with each other in nature. Such reassembly is particularly useful in producing "hybrid" antibodies capable of binding more than one antigen; and in producing "composite" immunoglobuins wherein heavy and light chains of different origins essentially damp out specificity. Third, by genetic manipulation, "chimeric" antibodies can be formed wherein, for example, the variable regions, correspond to the amino acid sequence from one mammalian model system, whereas the constant region mimics the amino acid sequence of another. Again, the derivation of these two mimicked sequences may be from different species. Fourth, also by genetic manipulation, "altered" antibodies with improved specificity and other characteristics can be formed.

Two other types of immunoglobulin-like moieties may be produced: "univalent" antibodies, which are useful as homing carriers to target tissues, and "Fab proteins" which include only the "Fab" region of an immunoglobulin molecule i.e, the branches of the "Y". These univalent antibodies and Fab fragments may also be "mammalian" i.e., mimic mammalian amino acid sequences; novel assemblies of mammalian chains, or chimeric, where for example, the constant and variable sequence patterns may be of different origin. Finally, either the light chain or heavy chain alone, or portions thereof, produced by recombinant techniques are included in the invention and may be mammalian or chimeric.

In other aspects, the invention is directed to DNA which encodes the aforementioned NSIs, antibodies, and portions thereof, as well as expression vectors or plasmids capable of effecting the production of such immunoglobulins in suitable host cells. It includes the host cells and cell cultures which result from transformation with these vectors. Finally, the invention is directed to methods of producing these NSIs and antibodies, and the DNA sequences, plasmids, and transformed cells intermediate to them.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a representation of the general structure of immunoglobulins.

FIG. **2A** and **2B** show the detailed sequence of the cDNA insert of pK17G4 which encodes kappa anti CEA chain.

FIG. **3** shows the coding sequence of the fragment shown in FIG. **2**, along with the corresponding amino acid sequence.

FIGS. **4A**, **4B** and **4C** show the combined detailed sequence of the cDNA inserts of pγ298 and pγ11 which encode gamma anti CEA chain.

FIGS. **5A** and **5B** show the corresponding amino acid sequence encoded by the fragment in FIG. **4**.

FIGS. **6** and **7** outline the construction of expression vectors for kappa and gamma anti-CEA chains respectively.

FIGS. **8A**, **8B**, and **8C** show the results of sizing gels run on extracts of *E. coli* expressing the genes for gamma chain, kappa chain, and both kappa and gamma chains respectively.

FIG. **9** shows the results of western blots of extracts of cells transformed as those in FIGS. **8**.

FIG. **10** shows a standard curve for ELISA assay of anti CEA activity.

FIGS. **11** and **12** show the construction of a plasmid for expression of the gene encoding a chimeric heavy chain.

FIG. **13** shows the construction of a plasmid for expression of the gene encoding the Fab region of heavy chain.

DETAILED DESCRIPTION

A. Definitions

As used herein, "antibodies" refers to tetramers or aggregates thereof which have specific immunoreactive activity, comprising light and heavy chains usually aggregated in the "Y" configuration of FIG. **1**, with or without covalent linkage between them; "immunoglobulins" refers to such assemblies whether or not specific immunoreactive activity is a property. "Non-specific immunoglobulin" ("NSI") means those immunoglobulins which do not possess specificity—i.e., those which are not antibodies.

"Mammalian antibodies" refers to antibodies wherein the amino acid sequences of the chains are homologous with those sequences found in antibodies produced by mammalian systems, either in situ, or in hybridomas. These antibodies antibodies mimic antibodies which are otherwise capable of being generated, although in impure form, in these traditional systems.

"Hybrid antibodies" refers to antibodies wherein chains are separately homologous with reference mammalian antibody chains and represent novel assemblies of them, so that two different antigens are precipitable by the tetramer. In hybrid antibodies, one pair of heavy and light chain is homologous to antibodies raised against one antigen, while the other pair of heavy and light chain is homologous to those raised against another antigen. This results in the property of "divalence" i.e., ability to bind two antigens simultaneously. Such hybrids may, of course, also be formed using chimeric chains, as set forth below.

"Composite" immunoglobulins means those wherein the heavy and light chains mimic those of different species origins or specificities, and the resultant is thus likely to be a non-specific immunoglobulin (NSI), i.e.—lacking in antibody character.

"Chimeric antibodies" refers to those antibodies wherein one portion of each of the amino acid sequences of heavy and light chains is homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular class, while the remaining segment of the chains is homologous to corresponding sequences in another. Typically, in these chimeric antibodies, the variable region of both light and heavy chains mimics the variable regions of antibodies derived from one species of mammals, while the constant portions are homologous to the sequences in antibodies derived from another. One clear advantage to such chimeric forms is that, for example, the variable regions can conveniently be derived from presently known sources using readily available hybridomas or B cells from non human host organisms in combination with constant regions derived from, for example, human cell preparations. While the variable region has the advantage of ease of preparation, and the specificity is not affected by its source, the constant region being human, is less likely to elicit an immune response from

a human subject when the antibodies are injected than would the constant region from a non-human source.

However, the definition is not limited to this particular example. It includes any antibody in which either or both of the heavy or light chains are composed of combinations of sequences mimicking the sequences in antibodies of different sources, whether these sources be differing classes, differing antigen respones, or differing species of origin and whether or not the fusion point is at the variable/constant boundary. Thus, it is possible to produce antibodies in which neither the constant nor the variable region mimic known antibody sequences. It then becomes possible, for example, to construct antibodies whose variable region has a higher specific affinity for a particular antigen, or whose constant region can elicit enhanced complement fixation or to make other improvements in properties possessed by a particular constant region.

"Altered antibodies" means antibodies wherein the amino acid sequence has been varied from that of a mammalian or other vertebrate antibody. Because of the relevance of recombinant DNA techniques to this invention, one need not be confined to the sequences of amino acids found in natural antibodies; antibodies can be redesigned to obtain desired characteristics. The possible variations are many and range from the changing of just one or a few amino acids to the complete redesign of, for example, the constant region. Changes in the constant region will, in general, be made in order to improve the cellular process characteristics, such as complement fixation, interaction with membranes, and other effector functions. Changes in the variable region will be made in order to improve the antigen binding characteristics. The antibody can also be engineered so as to aid the specific delivery of a toxic agent according to the "magic bullet" concept. Alterations, can be made by standard recombinant techniques and also by oligonucleotide-directed mutagenesis techniques (Dalbadie-McFarland, et al *Proc. Natl. Acad. Sci.(USA)*, 79:6409 (1982)).

"Univalent antibodies" refers to aggregations which comprise a heavy chain/light chain dimer bound to the Fc (or stem) region of a second heavy chain. Such antibodies are specific for antigen, but have the additional desirable property of targeting tissues with specific antigenic surfaces, without causing its antigenic effectiveness to be impaired—i.e., there is no antigenic modulation. This phenomenon and the property of univalent antibodies in this regard is set forth in Glennie, M. J., et al., *Nature*, 295: 712 (1982). Univalent antibodies have heretofore been formed by proteolysis.

"Fab" region refers to those portions of the chains which are roughly equivalent, or analogous, to the sequences which comprise the Y branch portions of the heavy chain and to the light chain in its entirety, and which collectively (in aggregates) have been shown to exhibit antibody activity. "Fab protein", which protein is one of the aspects of the invention, includes aggregates of one heavy and one light chain (commonly known as Fab'), as well as tetramers which correspond to the two branch segments of the antibody Y, (commonly known as $F(ab)_2$), whether any of the above are covalently or non-covalently aggregated, so long as the aggregation is capable of selectively reacting with a particular antigen or antigen family. Fab antibodies have, as have univalent ones, been formed heretofore by proteolysis, and share the property of not eliciting antigen modulation on target tissues. However, as they lack the "effector" Fc portion they cannot effect, for example, lysis of the target cell by macrophages.

"Fab protein" has similar subsets according to the definition of the present invention as does the general term "antibodies" or "immunoglobulins". Thus, "mammalian" Fab protein, "hybrid" Fab protein "chimeric" Fab and "altered" Fab protein are defined analogously to the corresponding definitions set forth in the previous paragraphs for the various types of antibodies.

Individual heavy or light chains may of course be "mammalian", "chimeric" or "altered" in accordance with the above. As will become apparent from the detailed description of the invention, it is possible, using the techniques disclosed to prepare other combinations of the four-peptide chain aggregates, besides those specifically defined, such as hybrid antibodies containing chimeric light and mammalian heavy chains, hybrid Fab proteins containing chimeric Fab proteins of heavy chains associated with mammalian light chains, and so forth.

"Expression vector" includes vectors which are capable of expressing DNA sequences contained therein, i.e., the coding sequences are operably linked to other sequences capable of effecting their expression. It is implied, although not always explicitly stated, that these expression vectors must be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. Clearly a lack of replicability would render them effectively inoperable. A useful, but not a necessary, element of an effective expression vector is a marker encoding sequence—i.e. a sequence encoding a protein which results in a phenotypic property (e.g. tetracycline resistance) of the cells containing the protein which permits those cells to be readily identified. In sum, "expression vector" is given a functional definition, and any DNA sequence which is capable of effecting expression of a specified contained DNA code is included in this term, as it is applied to the specified sequence. As at present, such vectors are frequently in the form of plasmids, thus "plasmid" and "expression vector" are often used interchangeably. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which may, from time to time become known in the art.

"Recombinant host cells" refers to cells which have been transformed with vectors constructed using recombinant DNA techniques. As defined herein, the antibody or modification thereof produced by a recombinant host cell is by virtue of this transformation, rather than in such lesser amounts, or more commonly, in such less than detectable amounts, as would be produced by the untransformed host.

In descriptions of processes for isolation of antibodies from recombinant hosts, the terms "cell" and "cell culture" are used interchangeably to denote the source of antibody unless it is clearly specified otherwise. In other words, recovery of antibody from the "cells" may mean either from spun down whole cells, or from the cell culture containing both the medium and the suspended cells.

B. Host Cell Cultures and Vectors

The vectors and methods disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms.

In general, of course, prokaryotes are preferred for cloning of DNA sequences in constructing the vectors useful in the invention. For example, *E. coli* K12 strain

294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include *E. coli* strains such as *E. coli* B, and *E. coli* X1776 (ATTC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes may also be used for expression. The aforementioned strains, as well as *E. coli* W3110 (F^{31}, λ^-, prototrophic, ATTC No. 27325), bacilli such as *Bacillus subtilus*, and other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various *Pseudomonas* species may be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (Bolivar, et al., *Gene* 2: 95 (1977)). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins. Those promoters most commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems (Chang et al, *Nature*, 275: 615 (1978); Itakura, et al, *Science*, 198: 1056 (1977); (Goeddel, et al *Nature* 281: 544 (1979)) and a tryptophan (trp) promoter system (Goeddel, et al, *Nucleic Acids Res.*, 8: 4057 (1980); EPO Appl Publ No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebenlist, et al, *Cell* 20: 269 (1980)).

In addition to prokaryates, eukaryotic microbes, such as yeast cultures may also be used. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example, (Stinchcomb, et al, *Nature*, 282: 39 (1979); Kingsman et al, *Gene*, 7: 141 (1979); Tschemper, et al, *Gene*, 10: 157 (1980)) is commonly used. This plasmid already contains the trpl gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, *Genetics*, 85: 12 (1977)). The presence of the trpl lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman, et al., *J. Biol. Chem.*, 255: 2073 (1980)) or other glycolytic enzymes (Hess, et al, *J. Adv. Enzyme Reg.*, 7: 149 (1968); Holland, et al, *Biochemistry*, 17: 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, an enzymes responsible for maltose and galactose utilization (Holland, ibid.). Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However interest has been greatest in vertebrate cells, and propogation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (*Tissue Culture*, Academic Pres, Kruse and Patterson, editors (1973)). Examlples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers, et al, *Nature*, 273: 113 (1978)) incorporated herein by reference. Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g. Polyoma, Adeno, VSV, BPV, etc.) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

It will be understood that this invention, although described herein in terms of a preferred embodiment, should not be construed as limited to those host cells, vectors and expression systems exemplified.

C. Methods Employed

C.1 Transformation

If cells without formidable cell wall barriers are used as host cells, transfection is carried out by the calcium phosphate precipitation method as described by Graham and Van der Eb, *Virology*, 52: 546 (1978). However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used.

If prokaryotic cells or cells which contain substantial cell wall constructions are used, the preferred method

of transfection is calcium treatment using calcium chloride as described by Cohen, F. N. et al *Proc. Natl. Acad. Sci. (USA)*, 69: 2110 (1972).

C.2 Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required. The methods employed are not dependent on the DNA source, or intended host.

Cleavage is performed by treating with restriction enzyme (or enzymes) in suitable buffer. In general, about 1 μg plasmid or DNA fragments is used with about 1 unit of enzyme in about 20 μl of buffer solution. (Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer.) Incubation times of about 1 hour at 37° C. are workable. After incubations, protein is removed by extraction with phenol and chloroform, and the nucleic acid is recovered from the aqueous fraction by precipitation with ethanol.

If blunt ends are required, the preparation is treated for 15 minutes at 15° with 10 units of *E. coli* DNA Polymerase I (Klenow), phenol-chloroform extracted, and ethanol precipitated.

Size separation of the cleaved fragments is performed using 6 percent polyacrylamide gel described by Goeddel, D., et al, *Nucleic Acids Res.*, 8: 4057 (1980) incorporated herein by reference.

For ligation, approximately equimolor amounts of the desired components, suitably end tailored to provide correct matching are treated with about 10 units T4 DNA ligase per 0.5 μg DNA. (When cleaved vectors are used as components, it may be useful to prevent religation of the cleaved vector by pretreatment with bacterial alkaline phosphatase.)

In the examples described below correct ligations for plasmid construction are confirmed by transforming *E. coli* K12 strain 294 (ATCC 31446) with the ligation mixture. Successful transformants were selected by ampicillin or tetracycline resistance depending on the mode of plasmid construction. Plasmids from the transformants were then prepared, analyzed by restriction and/or sequenced by the method of Messing, et al, *Nucleic Acids Res.*, 9: 309 (1981) or by the method of Maxam, et al, *Methods in Enzymology*, 65: 499 (1980).

D. Outline of Procedures

D.1 Mammalian Antibodies

The first type of antibody which forms a part of this invention, and is prepared by the methods thereof, is "mammalian antibody"-one wherein the heavy and light chains mimic the amino acid sequences of an antibody otherwise produced by a mature mammalian B lymphocyte either in situ or when fused with an immortalized cell as part of a hybridoma culture. In outline, these antibodies are produced as follows:

Messenger RNA coding for heavy or light chain is isolated from a suitable source, either mature B cells or a hybridoma culture, employing standard techniques of RNA isolation, and the use of oligo-dT cellulose chromatography to segregate the poly-A mRNA.. The poly-A mRNA may, further, be fractionated to obtain sequences of sufficient size to code for the amino acid sequences in the light or heavy chain of the desired antibody as the case may be.

A cDNA library is then prepared from the mixture of mRNA using a suitable primer, preferably a nucleic acid sequence which is characteristic of the desired cDNA. Such a primer may be hypothesized and synthesized based on the amino acid sequence of the antibody if the sequence is known. In the alternative cDNA from unfractionated poly-A mRNA from a cell line producing the desired antibody or poly-dT may also be used. The resulting cDNA is optionally size fractionated on polyacrylamide gel and then extended with, for example, dC residues for annealing with pBR322 or other suitable cloning vector which has been cleaved by a suitable restriction enzyme, such as Pst I, and extended with dG residues. Alternative means of forming cloning vectors containing the cDNA using other tails and other cloning vector remainder may, of course, also be used but the foregoing is a standard and preferable choice. A suitable host cell strain, typically *E. coli*, is transformed with the annealed cloning vectors, and the successful transformants identified by means of, for example, tetracycline resistance or other phenotypic characteristic residing on the cloning vector plasmid.

Successful transformants are picked and transferred to microtiter dishes or other support for further growth and preservation. Nitrocellulose filter imprints of these growing cultures are then probed with suitable nucleotide sequences containing bases known to be complementary to desired sequences in the cDNA. Several types of probe may be used, preferably synthetic single stranded DNA sequences labeled by kinasing with ATP^{32}. The cells fixed to the nitrocellulose filter are lysed, the DNA denatured, and then fixed before reaction with kinased probe. Clones which successfully hybridize are detected by contact with a photoplate, then plasmids from the growing colonies isolated and sequenced by means known in the art to verify that the desired portions of the gene are present.

The desired gene fragments are excised and tailored to assure appropriate reading frame with the control segments when inserted into suitable expression vectors. Typically, nucleotides are added to the 5' end to include a start signal and a suitably positioned restriction endonuclease site.

The tailored gene sequence is then positioned in a vector which contains a promoter in reading frame with the gene and compatible with the proposed host cell. A number of plasmids such as those described in U.S. Pat. Nos. 4,663,283 and 4,456,748 and pending U.S. Ser. No. 291,892, have been described which already contain the appropriate promoters, control sequences, ribosome binding sites, and transcription termination sites, as well as convenient markers.

In the present invention, the gene coding for the light chain and that coding for the heavy chain are recovered separately by the procedures outlined above. Thus they may be inserted into separate expression plasmids, or together in the same plasmid, so long as each is under suitable promoter and translation control.

The expression vectors constructed above are then used to transform suitable cells. The light and heavy chains may be transformed into separate cell cultures, either of the same or of differing species; separate plasmids for light and heavy chain may be used to co-transform a single cell culture, or, finally, a single expression plasmid containing both genes and capable of expressing the genes for both light and heavy chain may be transformed into a single cell culture.

Regardless of which of the three foregoing options is chosen, the cells are grown under conditions appropriate to the production of the desired protein. Such condi-

tions are primarily mandated by the type of promoter and control systems used in the expression vector, rather than by the nature of the desired protein. The protein thus produced is then recovered from the cell culture by methods known in the art, but choice of which is necessarily dependent on the form in which the protein is expressed. For example, it is common for mature heterologous proteins expressed in *E. coli* to be deposited within the cells as insoluble particles which require cell lysis and solubilization in denaturant to permit recovery. On the other hand, proteins under proper synthesis circmstances, in yeast and bacterial strains, can be secreted into the medium (yeast and gram positive bacteria) or into the periplasmic space (gram negative bacteria) allowing recovery by less drastic procedures. Tissue culture cells as hosts also appear, in general, to permit reasonably facile recovery of heterologous proteins.

When heavy and light chain are coexpressed in the same host, the isolation procedure is designed so as to recover reconstituted antibody. This can be accomplished in vitro as described below, or might be possible in vivo in a microorganism which secretes the IgG chains out of the reducing environment of the cytoplasm. A more detailed description is given in D.2, below.

D.2 Chain Recombination Techniques

The ability of the method of the invention to produce heavy and light chains or portions thereof, in isolation from each other offers the opportunity to obtain unique and unprecedented assemblies of immunoglobulins, Fab regions, and univalent antibodies. Such preparations require the use of techniques to reassemble isolated chains. Such means are known in the art, and it is, thus, appropriate to review them here.

While single chain disulfide bond containing proteins have been reduced and reoxidized to regenerate in high yield native structure and activity (Freedman, R. B., et al. In *Enzymology of Post Translational Modification of Proteins*, I: 157-212 (1980) Academic Press, NY.), proteins which consist of discontinuous polypeptide chains held together by disulfide bonds are more difficult to reconstruct in vitro after reductive cleavage. Insulin, a cameo case, has received much experimental attention over the years, and can now be reconstructed so efficiently that an industrial process has been built around it (Chance, R. E., et al., In *Peptides: Proceedings of the Seventh Annual American Peptide Symposium* (Rich, D. H. and Gross, E., eds.) 721-728, Pierce Chemical Co., Rockford, IL. (1981)).

Immunoglobulin has proved a more difficult problem than insulin. The tetramer is stabilized intra and intermolecularly by 15 or more disulfide bonds. It has been possible to recombine heavy and light chains, disrupted by cleavage of only the interchain disulfides, to regain antibody activity even without restoration of the interchain disulfides (Edelman, G. M., et al., *Proc. Natl. Acad. Sci (USA)* 50: 753 (1963)). In addition, active fragments of IgG formed by proteolysis (Fab fragments of ~50,000 MW) can be split into their fully reduced heavy chain and light chain components and fairly efficiently reconstructed to give active antibody (Haber, E., *Proc. Natl. Acad. Sci. (USA)* 52: 1099 (1964); Whitney, P. L., et al., *Proc. Natl. Acad. Sci. (USA)* 53: 524 (1965)). Attempts to reconstitute active antibody from fully reduced native IgG have been largely unsuccessful, presumably due to insolubility of the reduced chains and of side products or intermediates in the refolding pathway (see discussion in Freedman, M. H., et al., *J. Biol. Chem.* 241: 5225 (1966)). If, however, the immunoglobulin is randomly modified by polyalanylation of its lysines before complete reduction, the separated chains have the ability to recover antigen-combining activity upon reoxidation (ibid).

A particularly suitable method for immunoglobulin reconstitution is derivable from the now classical insulin recombination studies, wherein starting material was prepared by oxidative sulfitolysis, thus generating thiol-labile S-sulfonate groups at all cysteines in the protein, non-reductively breaking disulfides (Chance et al. (supra)). Oxidative sulfitolysis is a mild disulfide cleavage reaction (Means, G. E., et al., *Chemical Modification of Proteins*, Holden-Day, San Francisco (1971) which is sometimes more gentle than reduction (Wetzel, R., *Biochemistry*, submitted (1983)), and which generates derivatives which are stable until exposed to mild reducing agent at which time disulfide reformation can occur via thiol-disulfide interchange (Morehead, H., et al. *Biochemistry*, in press, (1983)). In the present invention the heavy and light chain S-sulfonates generated by oxidative sulfitolysis were reconstituted utilizing both air oxidation and thiol-disulfide interchange to drive disulfide bond formation. The general procedure is set forth in detail in U.S. Pat. No. 4,599,197 incorporated herein by reference.

D.3 Variants Permitted by Recombinant Technology

Using the techniques described in paragraphs D.1 and D.2, additional operations which were utilized to gain efficient production of mammalian antibody can be varied in quite straightforward and simple ways to produce a great variety of modifications of this basic antibody form. These variations are inherent in the use of recombinant technology, which permits modification at a genetic level of amino acid sequences in normally encountered mammalian immunoglobulin chains, and the great power of this approach lies in its ability to achieve these variations, as well as in its potential for economic and specific production of desired scarce, and often contaminated, molecules. The variations also inhere in the ability to isolate production of individual chains, and thus create novel assemblies.

Briefly, since genetic manipulations permit reconstruction of genomic material in the process of construction of expression vectors, such reconstruction can be manipulated to produce new coding sequences for the components of "natural" antibodies or immunoglobulins. As discussed in further detail below, the coding sequence for a mammalian heavy chain may not be derived entirely from a single source or a single species, but portions of a sequence can be recovered by the techniques described in D.1 from differing pools of mRNA, such as murine-murine hybridomas, human-murine hybridomas, or B cells differentiated in response to a series of antigen challenges. The desired portions of the sequences in each case can be recovered using the probe and analysis techniques described in D.1, and recombined in an expression vector using the same ligation procedures as would be employed for portions of the same model sequence. Such chimeric chains can be constructed of any desired length; hence, for example, a complete heavy chain can be constructed, or only sequence for the Fab region thereof.

The additional area of flexibility which arises from the use of recombinant techniques results from the power to produce heavy and light chains or fragments thereof in separate cultures or of unique combinations

of heavy and light chain in the same culture, and to prevent reconstitution of the antibody or immunoglobulin aggregation until the suitable components are assembled. Thus, while normal antibody production results automatically in the formation of "mammalian antibodies" because the light and heavy chain portions are constructed in response to a particular determinant in the same cell, the methods of the present invention present the opportunity to assemble entirely new mixtures. Somewhat limited quantities of "hybrid" antibodies have been produced by "quadromas" i.e., fusions of two hybridoma cell cultures which permit random assemblies of the heavy and light chains so produced.

The present invention permits a more controlled assembly of desired chains, either by mixing the desired chains in vitro, or by transforming the same culture with the coding sequences for the desired chains.

D.4 Composite Immunoglobulins

The foregoing procedure, which describes in detail the recombinant production of mammalian antibodies is employed with some modifications to construct the remaining types of antibodies or NSIs encompassed by the present invention. To prepare the particular embodiment of composite non-specific immunoglobulin wherein the homology of the chains corresponds to the sequences of immunoglobulins of different specificities, it is of course, only necessary to prepare the heavy and light chains in separate cultures and reassemble them as desired.

For example, in order to make an anti-CEA light chain/anti-hepatitis heavy chain composite antibody, a suitable source for the mRNA used as a template for the light chain clone would comprise, for instance, the anti CEA producing cell line of paragraph E.1. The mRNA corresponding to heavy chain would be derived from B cells raised in response to hepatitis infection or from hybridoma in which the B cell was of this origin. It is clear that such composites can be assembled using the methods of the invention almost at will, and are limited only by available sources of mRNA suitable for use as templates for the respective chains. All other features of the process are similar to those described above.

D.5 Hybrid Antibodies

Hybrid antibodies are particularly useful as they are capable of simultaneous reaction with more than one antigen. Pairs of heavy and light chains corresponding to chains of antibodies for different antigens, such as those set forth in paragraph D.4 are prepared in four separate cultures, thus preventing premature assembly of the tetramer. Subsequent mixing of the four separately prepared peptides then permits assembly into the desired tetramers. While random aggregation may lead to the formation of considerable undesired product, that portion of the product in which homologous light and heavy chains are bound to each other and mismatched to another pair gives the desired hybrid antibody.

D.6 Chimeric Antibodies

For construction of chimeric antibodies (wherein, for example, the variable sequences are separately derived from the constant sequences) the procedures of paragraph D.1 and D.2 are again applicable with appropriate additions and modifications. A preferred procedure is to recover desired portions of the genes encoding for parts of the heavy and light chains from suitable, differing, sources and then to religate these fragments using restriction endonucleases to reconstruct the gene coding for each chain.

For example, in a particular preferred chimeric construction, portions of the heavy chain gene and of the light chain gene which encode the variable sequences of antibodies produced by a murine hybridoma culture are recovered and cloned from this culture and gene fragments encoding the constant regions of the heavy and light chains for human antibodies recovered and cloned from, for example, human myeloma cells. Suitable restriction enzymes may then be used to ligate the variable portions of the mouse gene to the constant regions of the human gene for each of the two chains. The chimeric chains are produced as set forth in D.1, aggregated as set forth in D.2 and used in the same manner as the non-chimeric forms. Of course, any splice point in the chains can be chosen.

D.7 Altered Antibodies

Altered antibodies present, in essence, an extension of chimeric ones. Again, the techniques of D.1 and D.2 are applicable; however, rather than splicing portions of the chain(s), suitable amino acid alterations, deletions or additions are made using available techniques such as mutagenesis (supra). For example, genes which encode antibodies having diminished complement fixation properties, or which have enhanced metal binding capacities are prepared using such techniques. The latter type may, for example, take advantage of the known gene sequence encoding metalothionein II (Karin, M., et al., *Nature*, 299: 797 (1982)). The chelating properties of this molecular fragment are useful in carrying heavy metals to tumor sites as an aid in tumor imaging (Scheinberg, D. A., et al., *Science*, 215: 19 (1982).

D.8 Univalent Antibodies

In another preferred embodiment, antibodies are formed which comprise one heavy and light chain pair coupled with the Fc region of a third (heavy) chain. These antibodies have a particularly useful property. They can, like ordinary antibodies, be used to target antigenic surfaces of tissues, such as tumors, but, unlike ordinary antibodies, they do not cause the antigenic surfaces of the target tissue to retreat and become non-receptive. Ordinary antibody use results in aggregation and subsequent inactivation, for several hours, of such surface antigens.

The method of construction of univalent antibodies is a straightforward application of the invention. The gene for heavy chain of the desired Fc region is cleaved by restriction enzymes, and only that portion coding for the desired Fc region expressed. This portion is then bound using the technique of D.2 to separately produced heavy chain the desired pairs separated from heavy/heavy and Fc/Fc combinations, and separately produced light chain added. Pre-binding of the two heavy chain portions thus diminishes the probability of formaion of ordinary antibody.

D.9 Fab Protein

Similarly, it is not necessary to include the entire gene for the heavy chain portion. All of the aforementioned variations can be superimposed on a procedure for Fab protein production and the overall procedure differs only in that that portion of the heavy chain coding for the amino terminal 220 amino acids is employed in the appropriate expression vector.

E. Specific Examples of Preferred Embodiments

The invention has been described above in general terms and there follow several specific examples of embodiments which set forth details of experimental procedure in producing the desired antibodies. Example E.1 sets forth the general procedure for preparing anti

CEA antibody components, i.e. for a "mammalian antibody". Example E.3 sets forth the procedure for reconstitution and thus is applicable to preparation of mammalian, composite, hybrid and chimeric immunoglobulins, and Fab proteins and univalent antibodies. Example E.4 sets forth the procedure for tailoring the heavy or light chain so that the variable and constant regions may be derived from different sources. Example E.5 sets forth the method of obtaining a shortened heavy chain genome which permits the production of the Fab regions and, in an analogous manner, Fc region.

The examples set forth below are included for illustrative purposes and do not limit the scope of the invention.

E.1 Construction of Expression Vectors for Murine anti-CEA Antibody Chains and Peptide Synthesis

Carcinoembryonic antigen (CEA) is associated with the surface of certain tumor cells of human origin (Gold, P., et al., *J. Exp. Med.*, 122: 467 (1965)). Antibodies which bind to CEA (anti-CEA antibodies) are useful in early detection of these tumors (Van Nagell, T. R., et al., *Cancer Res.* 40: 502 (1980)), and have the potential for use in treatment of those human tumors which appear to support CEA at their surfaces. A mouse hybridoma cell line which secretes anti-CEA antibodies of the $Ig\gamma_1$ class, CEA.66-E3, has been prepared as described by Wagener, C, et al., *J. Immunol.* (in press) which is incorporated herein by reference, and was used as mRNA source. The production of anti CEA antibodies by this cell line was determined. The N-terminal sequences of the antibodies produced by these cells was compared with those of monoclonal anti CEA as follows. Purified IgG was treated with PCAse (Podell, D. N., et al., BBRC 81: 176 (1978)), and then dissociated in 6M guanidine hydrochloride, 10 mM 2-mercaptoethanol (1.0 mg of immunoglobulin, 5 min, 100° C. water bath). The dissociated chains were separated on a Waters Associates alkyl phenyl column using a liner gradient from 100 percent A (0.1 percent TFA-water) to 90 percent B (TFA/H_2O/MeCN 0.1/9.9/90) at a flow rate of 0.8 ml/min. Three major peaks were eluted and analyzed on SDS gels by silver staining. The first two peaks were pure light chain (MW 25,000 daltons), the third peak showed a (7:3) mixture of heavy and light chain. 1.2 nmoles of light chain were sequenced by the method of Shively, J. E., *Methods in Enzymology*, 79: 31 (1981), with an NH_2-terminal yield of 0.4 nmoles. A mixture of heavy and light chains (3 nmoles) was also sequenced, and sequence of light chain was deducted from the double sequence to yield the sequence of the heavy chain.

In the description which follows, isolation and expression of the genes for the heavy and light chains for anti CEA antibody produced by CEA.66-E3 are described. As the constant regions of these chains belong to the gamma and kappa families, respectively, "light chain" and "kappa chain", and "heavy chain" and "gamma chain", respectively, are used interchangeably below.

E.1.1 Isolation of Messenger RNA for Anti CEA Light and Heavy (Kappa and Gamma) Chains

Total RNA for CEA.66-E3 cells was extracted essentially as reported by Lynch et al, *Virology*, 98: 251 (1979). Cells were pelleted by centrifugation and approximately 1 g portions of pellet resuspended in 10 ml of 10 mM NaCl, 10 mM Tris HCl (pH 7.4), 1.5 mM $MgCl_2$. The resuspended cells were lysed by addition of non-ionic detergent NP-40 to a final concentration of 1 percent, and nuclei removed by centrifugation. After addition of SDS (pH 7.4) to 1 percent final concentration, the supernatant was extracted twice with 3 ml portions of phenol (redistilled)/chloroform:isoamyl alcohol 25:1 at 4° C. The aqueous phase was made 0.2M in NaCl and total RNA was precipitated by addition of two volumes of 100 percent ethanol and overnight storage at −20° C. After centrifugation, polyA mRNA was purified from total RNA by oligo-dT cellulose chromatography as described by Aviv and Leder, *Proc. Nat'l. Acad. Sci (USA)*, 69: 1408 (1972). 142 μg of polyA mRNA was obtained from 1 g cells.

E.1.2 Preparation of *E. coli* Colony Library Containing Plasmids with Heavy and Light DNA Sequence Inserts

5 μg of the unfractionated polyA mRNA prepared in paragraph E.1.1 was used as template for oligo-dT primed preparation of double-stranded (ds) cDNA by standard procedures as described by Goeddel et al., Nature 281: 544 (1979) and Wickens et al., *J. Biol. Chem.* 253: 2483 (1978) incorporated herein by reference. The cDNA was size fractionated by 6 percent polyacrylamide gel electrophoresis and 124 ng of ds cDNA greater than 600 base pairs in length was recovered by electroelution. A 20 ng portion of ds cDNA was extended with deoxy C residues using terminal deoxynucleotidyl transferase as described in Chang et al., *Nature* 275: 617 (1978) incorporated herein by reference, and annealed with 200 ng of the plasmid pBR322 (Bolivar et al., *Gene* 2: 95 (1977)) which had been cleaved with Pst I and tailed with deoxy G. Each annealed mixture was then transformed into *E. coli* K12 strain 294 (ATCC No. 31446). Approximately 8500 ampicillin sensitive, tetracycline resistant transformants were obtained.

E.1.3 Preparation of Synthetic Probes

The 14 mer, 5'GGTGGGAAGATGGA 3' complementary to the coding sequence of constant region for mous MOPC21 kappa chain which begins 25 basepairs 3' of the variable region DNA sequence was used as kappa chain probe. A 15 mer, 5'GACCAGGCATCCCAG 3', complementary to a coding sequence located 72 basepairs 3' of the variable region DNA sequence for mouse MOPC21 gamma chain was used to probe gamma chain gene.

Both probes were synthesized by the phosphotriester method described in German Offenlegungschrift No. 2644432, incorporated herein by reference, and made radioactive by kinasing as follows: 250 ng of deoxyoligonucleotide were combined in 25 μl of 60 mM Tris HCl (pH 8), 10 mM $MgCl_2$, 15 mM beta-mercaptoethanol, and 100 μCi (γ-^{32}P) ATP (Amersham, 5000 Ci/mMole). 5 units of T4 polynucleotide kinase were added and the reaction was allowed to proceed at 37° C. for 30 minutes and terminated by additon of EDTA to 20 mM.

E.1.4 Screening of Colony Library for Kappa or Gamma Chain Sequences

~2000 colonies prepared as described in paragraph E.1.2 were individually inoculated into wells of microtitre dishes containing LB (Miller, Experiments in Molecular Genetics, p. 431-3, Cold Spring Harbor Lab., Cold Spring Harbor, N.Y. (1972))+5 μg/ml tetracycline and stored at −20° C. after addition of DMSO to 7 percent. Individual colonies from this library were transferred to duplicate sets of Schleicher and Schuell BA85/20 nitrocellulose filters and grown on agar plates containing LB+5 μg/ml tetracycline. After ~10 hours growth at 37° C. the colony filters

were transferred to agar plates containing LB+5 μg/ml tetracycline and 12.5 μg/ml chloramphicol and reincubated overnight at 37° C. The DNA from each colony was then denatured and fixed to the filter by a modification of the Grunstein-Hogness procedure as described in Grunstein et al., *Proc. Natl. Acad. Sci. (USA)* 72: 3961 (1975), incorporated herein by reference. Each filter was floated for 3 minutes on 0.5N NaOH, 1.5M NaCl to lyse the colonies and denature the DNA then neutralized by floating for 15 minutes on 3M NaCl, 0.5M Tris HCl (pH 7.5). The filters were then floated for an additional 15 minutes on 2×SSC, and subsequently baked for 2 hours in an 80° C. vacuum oven. The filters were prehybridized for ~2 hours at room temperature in 0.9M NaCl, 1× Denhardts, 100 mM Tris HCl (pH 7.5), 5 mM Na-EDTA, 1 mM ATP, 1M sodium phosphate (dibasic), 1 mM sodium pyrophosphate, 0.5 percent NP-40, and 200 μg/ml *E. coli* t-RNA, and hybridized in the same solution overnight, essentially as described by Wallace et al. *Nucleic Acids Research* 9: 879 (1981) using ~40×10^6 cpm of either the kinased kappa or gamma probe described above.

After extensive washing at 37° C. in 6X SSC, 0.1 percent SDS, the filters were exposed to Kodak XR-5 X-ray film with DuPont Lightning-Plus intensifying screens from 16–24 hours at −80° C. Approximately 20 colonies which hybridized with kappa chain probe and 20 which hybridized with gamma chain probe were characterized.

E.1.5 Characterization of Colonies which Hybridize to Kappa DNA Sequence Probe

Plasmid DNAs isolated from several different transformants which hybridized to kappa chain probe were cleaved with Pst I and fractionated by polyacrylamide gel electrophoresis (PAGE). This analysis demonstrated that a number of plasmid DNAs contained cDNA inserts large enough to encode full length kappa chain. The complete nucleotide sequence of the cDNA insert of one of these plasmids was determined by the dideoxynucleotide chain termination method as described by Smith, *Methods Enzymol.* 65, 560 (1980) incorporated herein by reference after subcloning restriction endonuclease cleavage fragments into M13 vectors (Messing et al., *Nucleic Acids Research* 9: 309 (1981). FIG. 2 shows the nucleotide sequence of the cDNA insert of pK17G4 and FIG. 3 shows the gene sequence with the corresponding amino acid sequence. Thus, the entire coding region of mouse anti-CEA kappa chain was isolated on this one large DNA fragment. The amino acid sequence of kappa chain, deduced from the nucleotide sequence of the pK17G4 cDNA insert, corresponds perfectly with the first 23 N-terminal amino acids of mature mouse anti-CEA kappa chain as determined by amino acid sequence analysis of purified mouse anti-CEA kappa chain. The coding region of pK17G4 contains 27 basepairs or 9 amino acids of the presequence and 642 basepairs or 214 amino acids of the mature protein. The mature unglycosylated protein (MW 24,553) has a variable region of 119 amino acids, including the J1 joining region of 12 amino acids, and a constant region of 107 amino acids. After the stop codon behind amino acid 215 begins 212 basepairs of 3' untranslated sequence up to the polyA addition. The kappa chain probe used to identify pK17G4 hybridizes to nucleotides 374–388 (FIG. 2).

E.1.6. Characterization of Colonies which Hybridize to Gamma 1 DNA Probe

Plasmid DNA isolated from several transformants positive for hybridization with the heavy chain gamma 1 probe was subjected to Pst I restriction endonuclease analysis as described in E.1.5. Plasmid DNAs demonstrating the largest cDNA insert fragments were selected for further study. Nucleotide sequence coding for mouse heavy (gamma-1) chain, shows an NcoI restriction endonuclease cleavage site near the junction between variable and constant region. Selected plasmid DNAs were digested with both PstI and NcoI and sized on polyacrylamide. This analysis allowed identification of a number of plasmid DNAs that contain NcoI restriction endonuclease sites, although none that demonstrate cDNA insert fragments large enough to encode the entire coding region of mouse anti-CEA heavy chain.

In one plasmid isolated, p γ298 the cDNA insert of about 1300 bp contains sequence information for the 5' untranslated region, the signal sequence and the N-terminal portion of heavy chain. Because pγ298 did not encode the C-terminal sequence for mouse anti-CEA gamma 1 chain, plasmid DNA was isolated from other colonies and screened with PstI and NcoI. The C-terminal region of the cDNA insert of pγ11 was sequenced and shown to contain the stop codon, 3' untranslated sequence and that portion of the coding sequence missing from p γ298.

FIG. 4 presents the entire nucleotide sequence of mouse anti-CEA heavy chain (as determined by the dideoxynucleotide chain termination method of Smith, *Methods Enzymol.*, 65: 560 (1980)) and FIG. 5 includes the translated sequence.

The amino acid sequence of gamma 1 (heavy chain) deduced from the nucleotide sequence of the pγ298 cDNA insert corresponds perfectly to the first 23 N-terminal amino acids of mature mouse anti-CEA gamma 1 chain as determined by amino acid sequence analysis of purified mouse anti-CEA gamma-1 chain. The coding region consists of 57 basepairs or 19 amino acids of presequences and 1346 basepairs or 447 amino acids of mature protein. The mature unglycosolated protein (MW 52,258) has a variable region of 135 amino acids, including a D region of 12 amino acids, and a J4 joining region of 13 amino acids. The constant region is 324 amino acids. After the stop codon behind amino acid 447 begins 96 bp of 3' untranslated sequences up to the polyA addition. The probe used to identify pγ298 and pγ11 hybridized to nucleotides 528–542 (FIG. 4).

E.1.7 Construction of a Plasmid For Direct Expression of Mouse Mature Anti-CEA Kappa Chain Gene, pKCEAtrp207-1*

FIG. 6 illustrates the construction of pKCEAtrp207-1*

First, an intermediate plasmid pHGH207-1*, having a single trp promoter, was prepared as follows:

The plasmid pHGH 207 (described in U.S. Pat. No. 4,663,283), was used to prepare pHGH 207-1, pHGH 207 was digested with BamHI, followed by partial digestion with EcoRI. The largest fragment, which contains the entire trp promoter, was isolated and ligated to the largest EcoRI-BamHI fragment from pBR322, and the ligation mixture used to transform *E. coli* 294. Tet and Amp resistant colonies were isolated, and most of them contained pHGH207-1. pHGH207-1* which lacks the EcoR1 site between the amp^R gene and the trp promoter, was obtained by partial digestion of pHGH207-1 (de Boer et al., *Promoters*, Rodriquez et al. Eds. (1982), pp. 462–481 U.S. Pat. No. 4,663,283) with

EcoR I, filling in the ends with Klenow and dNTPs, and religation.

5 μg of pHGH207-1* was digested with EcoRI, and the ends extended to blunt ends using 12 units of DNA Polymerase I in a 50 μl reaction containing 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4) and 1 mM in each dNTP at 37° C. for 1 hour, followed; by extraction with phenol/$CHCl_3$ and precipitation with ethanol. The precipitated DNA was digested with BamH I, and the large vector fragment (fragment 1) purified using 5 percent polyacrylamide gel electrophoresis, electroelution, phenol/$CHCl_3$ extraction and ethanol precipitation.

The DNA was resuspended in 50 μl of 10 mM Tris pH 8, 1 mM EDTA and treated with 500 units Bacterial Alkaline Phosphatase (BAP) for 30' at 65° followed by phenol/$CHCl_3$ extraction and ethanol precipitation.

A DNA fragment containing part of the light chain sequence was prepared as follows: 7 μg of pK17G4 DNA was digested with Pst I and the kappa chain containing cDNA insert was isolted by 6 percent gel electrophoresis, and electroelution. After phenol/$CHCl_3$ extraction, ethanol precipitaion and resuspensionin water, this fragment was digested with Ava II. The 333 bp Pst I-Ava II DNA fragment was isolated and purified from a 6 percent polyacrylamide gel.

A 15 nucleotide DNA primer was synthesized by the phosphotriester method G.O. 2,644,432 (supra) and has the following sequence:

Met Asp Ile Val Met
5' ATG GAC ATT GTT ATG 3'

The 5' methionine serves as the initiation codon. 500 ng of this primer was phosphorylated at the 5' end with 10 units T4 DNA kinase in 20 μl reaction containing 0.5 mM ATP. ~200 ng of the Pst I-Ava II DNA fragment was mixed with the 20 μl of the phosphorylated primer, heated to 95° C. for 3 minutes and quick frozen in a dry-ice ethanol bath. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C. this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, and digested to completion with Sau 3A. The reaction mixture was then electrophoresed on a 6 percent polyacrylamide gel and ~50 ng of the 182 basepair amino-terminal blunt-end to Sau 3A fragment (fragment 2) was obtained after electroelution.

100 ng of fragment 1 (supra) and 50 ng of fragment 2 were combined in 20 μl of 20 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 2.5 mM ATP and 1 unit of T4 DNA ligase. After overnight ligation at 14° C. the reaction was transformed into *E. coli* K12 strain 294. Restriction endonuclease digestion of plasmid DNA from a number of ampicillin resistant transformants indicated the proper construction and DNA sequene analysis proved the desired nucleotide sequence through the initiation codon of this new plasmid, pKCEAInt1 (FIG. 6).

The remainder of the coding sequence of the kappa light chain gene was prepared as follows:

The Pst I cDNA insert fragment from 7 μg of K17G4 DNA was partially digested with Ava II and the Ava II cohesive ends were extended to blunt ends in a DNA Polymerase I large fragment reaction. Following 6 percent polyacrylamide gel electrophoresis the 686 basepair Pst I to blunt ended Ava II DNA fragment was isolated, purified and subjected to Hpa II restriction endonuclease digestion. The 497 basepair Hpa II to blunt ended Ava II DNA fragment (fragment 3) was isoated and purified after gel electrophoresis.

10 μg of pKCEAInt1 DNA was digested with Ava I, extended with DNA polymerase I large fragment, and digested with Xba I. Both the large blunt ended Ava I to Xba I vector fragment and the small blunt ended Ava I to Xba I fragment were isolated and purified from a 6 percent polyacrylamide gel after electrophoresis. The large vector fragment (fragment 4) was treated with Bacterial Alkaline Phosphatase (BAP), and the small fragment was digested with Hpa II, electrophoresed on a 6 percent polyacrylamide and the 169 basepair Xba I-Hpa II DNA fragment (fragment 5) was purified. ~75 ng of fragment 4, ~50 ng of fragment 3 and ~50 ng of fragment 5 were combined in a T4 DNA ligase reactin and incubated overnight at 14°, and the reaction mixture transformed into *E. coli* K12 strain 294. Plasmid DNA from six ampicillin resistant transformants were analyzed by restriction endonuclease digestion. One plasmid DNA demonstrated the proper construction and was digested pKCEAInt2.

Final construction was effected by ligating the K-CEA fragment, including the trp promoter from pKCEAInt2 into pBR322(XAP). (pBR322(XAP) is prepared by deletion of the region of pBR322 between its unique AvaI and PvaII sites)

The K-CEA fragment was prepared by treating pKCEAInt2 with Ava I, blunt ending with DNA polymerase I (Klenow fragment) in the presence of DNTPs, digestion with Pst I and isolation of the desired fragment by gel electrophoresis and electroelution.

The large vector fragment from pBR322(XAP) was prepared by successive treatment with EcoR I, blunt ending with polymerase, and redigestion with Pst I, followed by isolation of the large vector fragment by electrophoresis and electroelution.

The K-CEA and large vector fragments as prepared in the preceding paragraphs were ligated with T4 DNA ligase, and the ligation mixture transformed into *E. coli* as above. Plasmid DNA from several ampicillin resistant transformants were selected for analysis, and one plasmid DNA demonstrated the proper construction, and was designated pKCEAtrp207-1*.

E.1.8 Construction of a Plasmid Vector for Direct Expression of Mouse Mature Anti-CEA Heavy (Gamma 1) Chain Gene, pγCEAtrp207-1*

FIG. 7 illustrates the construction of pγCEAtrp207-1*. This plasmid was constructed in two parts beginning with construction of the C-terminal regin of the gamma 1 gene.

5 μg of plasmid pHGH207-1* was digested with Ava I, extended to blunt ends with DNA polymerase I large fragment (Klenow fragment), extracted with phenol/$CHCl_3$, and ethanol precipitated. The DNA was digested with BamH I treated with BAP and the large fragment (fragment A) was purified by 6 percent polyacrylamide gel electrophoresis and electroelution.

~5 μg of pγ11 was digested with Pst I and the gamma chain cDNA insert fragment containing the C-terminal portion of the gene was purified, digested with Ava II followed by extension of the Ava II cohesive ends with Klenow, followed by Taq I digestion. The 375 basepair blunt ended Ava II to Taq I fragment

(fragment B) was isolated and purified by gel electrophoresis and electroelution.

9 μg of pγ298 was digested with Taq I and BamH I for isolation of the 496 basepair fragment (fragment C).

Approximately equimolar amounts of fragments A, B, and C were ligated overnight at 14° in 20 μl reaction mixture, then transformed into *E. coli* strain 294. The plasmid DNA from six ampicillin resistant transformants was committed to restriction endonuclease analysis and one plasmid DNA, named pγCEAInt, demonstrated the correct construction of the C-terminal portion of gamma 1 (FIG. 5).

To obtain the N-terminal sequences, 30 μg of pγ298 was digested with Pst I and the 628 basepair DNA fragment encoding the N-terminal region of mouse anti-CEA gamma chain was isolated and purified. This fragment was further digested with Alu I and Rsa I for isolation of the 280 basepair fragment. A 15 nucleotide DNA primer

met glu val met leu
5' ATG GAA GTG ATG CTG 3'

was synthesized by the phosphotriester method (supra).

The 5' methionine serves as the initiation codon. 500 ng of this synthetic oligomer primer was phosphorylated at the 5' end in a reaction with 10 units T4 DNA kinase containing 0.5 mM ATP in 20 μl reaction mixture. ~500 ng of the 280 basepair Alu I-Rsa I DNA fragment was mixed with the phosphorylated primer. The mixture was heat denatured for 3 minutes at 95° and quenched in dry-ice ethanol. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C., this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, and digested to completion with HpaII. ~50 ng of the expected 125 basepair blund-end to Hpa II DNA fragment (fragment D) was purified from the gel.

A second aliquot of pγ298 DNA was digested with Pst I, the 628 basepair DNA fragment purified by polyacrylamide gel electrophoresis, and further digested with BamH I ahd Hpa II. The resulting 380 basepair fragment (fragment E) was purified by gel electrophoresis.

~5 μg of pγCEAIntI was digested with EcoR I, the cohesive ends were made flush with DNA polymerase I (Klenow), further digested with BamH I, treated with BAP and electrophoresed on a 6 percent polyacrylamide gel. The large vector fragment (fragment F) was isolated and purified.

In a three fragment ligation, 50 ng fragment D, 100 ng fragment E, and 100 ng fragment F were ligated overnight at 4° in a 20 μreaction mixture and used to transform *E. coli* K12 strain 294. The plasmid DNAs from 12 ampicillin resistant transformants were analyzed for the correct construction and the nucleotide sequence surrounding the initiation codon was verified to be correct for the plasmid named pγCEAInt2.

The expression plasmid, pγCEAtrp207-1* used for expression of the heavy chain gene is prepared by a 3-way ligation using the large vector fragment from pBR322(XAP) (supra) and two fragments prepared from pγCEAInt2.

pBR322(XAP) was treated as above by digestion with EcoR1, blunt ending with DNA polymerase (Klenow) in the presence of dNTPs, followed by digestion with Pst I, and isolation of the large vector fragment by gel electrophoresis. A 1543 base pair fragment from pγCEAInt2 containing trp promoter linked with the N-terminal coding region of the heavy chain gene was isolated by treating pγCEAInt2 with Pst I followed by BamH I, and isolation of the desired fragment using PAGE. The 869 base pair fragment containing the C-terminal coding portion of the gene was prepared by partial digestion of pγCEAInt2 with Ava I, blund ending with Klenow, and subsequent digestion with BamH I, followed by purification of the desired fragment by gel electrophoresis.

The aforementioned three fragments were then ligated under standard conditions using T4 DNA ligase, and a ligation mixture used to transform *E. coli* strain 294. Plasmid DNAs from several tetracycline resistant transformants were analyzed; one plasmid DNA demonstrated the proper construction and was designated pγCEAtrp207-1*.

E.1.9. Production of Immunoglobulin Chains by *E. coli*

E. coli strain W3110 (ATTC No. 27325) was transformed with pγCEAtrp207-1* or pKCEAtrp207-1* using standard techniques.

To obtain double transformants, *E. coli* strain W3110 cells were transformed with a modified pKCEAtrp207-1*, pKCEAtrp207-1*Δ, which had been modified by cleaving a Pst I-Pvu I fragment from the amp^R gene and religating. Cells transformed with pKCEAtrp207-1*Δ are thus sensitive to ampicillin but still resistant to tetracycline. Successful transformants were retransformed using pγCEAInt2 which confers resistance to ampicillin but not tetracycline. Cells containing both pKCEAtrp207-1*Δ and pγCEAInt2 thus identified by growth in a medium containing both ampicillin and tetracycline.

To confirm the production of heavy and/or light chains in the transformed cells, the cell samples were inoculated into M9 tryptophan free medium containing 10 μg/ml tetracycline, and induced with indoleacrylic acid (IAA) when the OD 550 reads 0.5. The induced cells were grown at 37° C. during various time periods and then spun down, and suspended in TE buffer containing 2 percent SDS and 0.1M β-mercaptoethanol and boiled for 5 minutes. A 10× volume of acetone was added and the cells kept at 22° C. for 10 minutes, then centrifuged at 12,000 rpm. The precipitate was suspended in O'Farrell SDS sample buffer (O'Farrell, P. H., *J. Biol. Chem.*, 250: 4007 (1975)); boiled 3 minutes, recentrifuged, and fractionated using SDS PAGE (10 percent), and stained with silver stain (Goldman, D. et al., *Science* 211: 1437 (1981)); or subjected to Western blot using rabbit anti-mouse IgG (Burnett, W. N., et al., *Anal. Biochem.* 112: 195 (1981)), for identification light chain and heavy chain.

Cells transformed with pγCEAtrp207-1* showed bands upon SDS PAGE corresponding to heavy chain molecular weight as developed by silver stain. Cells transformed with pKCEAtrp207-1* showed the proper molecular weight band for light chain as identified by Western blot; double transformed cells showed bands for both heavy and light chain molecular weight proteins when developed using rabbit anti-mouse IgG by Western blot. These results are shown in FIGS. 8A, 8B, and 8C.

FIG. 8A shows results developed by silver stain from cells transformed with pγCEAtrp207-1*. Lane 1 is

monoclonal anti-CEA heavy chain (standard) from CEA.66-E3. Lanes 2b–5b are timed samples 2 hrs, 4 hrs, 6 hrs, and 24 hrs after IAA addition. Lanes 2a–5a are corresponding untransformed controls; Lanes 2c–5c are corresponding uninduced transformants.

FIG. **8B** shows results developed by Western blot from cells transformed with pKCEAtrp207-1*. Lanes 1b–6b are extracts from induced cells immediately, 1 hr, 3.5 hrs, 5 hrs, 8 hrs, and 24 hrs after IAA addition, and 1a–6a corresponding uninduced controls. Lane 7 is an extract from a pγCEAtrp207-1* control, lanes 8, 9, and 10 are varying amounts of anti CEA-kappa chain from CEA.66-E3 cells.

FIG. **8C** shows results developed by Western blot from four colonies of double tranformed cells 24 hours after IAA addition (lanes 4–7). Lanes 1–3 are varying amounts of monoclonal gamma chain controls, lanes 8 and 9 are untransformed and pγCEAtrp207-1* transformed cell extracts, respectively.

In another quantitative assay, frozen, transformed *E. coli* cells grown according to E.1.10 (below) were lysed by heating in sodium dodecyl sulfate (SDS)/β-mercaptoethanol cell lysis buffer at 100°. Aliquots were loaded on an SDS polyacrylamide gel next to lanes loaded with various amounts of hybridoma anti-CEA. The gel was developed by the Western blot, Burnett (supra), using ^{125}I-labeled sheep anti-mouse IgG antibody from New England Nuclear. The results are shown in FIG. **9**. The figure shows that the *E. coli* products co-migrate with the authentic hybridoma chains, indicating no detectable proteolytic degradation in *E. coli*. Heavy chain from mammalian cells is expected to be slightly heavier than *E. coli* material due to glycosylation in the former. Using the hybridoma lanes as a standard, the following estimates of heavy and light chain production were made:

	(Per gram of cells)
E. coli (W3110/pγCEAtrp207-1*)	5 mg γ
E. coli (W3110/pKCEAtrp207-1*)	1.5 mg K
E. coli (W3110/pKCEAtrp207-1*Δ, pγCEA Int2)	0.5 mg K, 1.0 mg γ

E.1.10 Reconstitution of Antibody from Recombinant K and Gamma Chains

In order to obtain heavy and light chain preparation for reconstitution, transformed cells were grown in larger batches, harvested and frozen. Conditions of growth of the variously transformed cells were as follows:

E. coli (W3110/pγCEAtrp207-1*) were inoculated into 500 ml LB medium containing 5 μg/ml tetracycline and grown on a rotary shaker for 8 hours. The culture was then transformed to 10 liters of fermentation medium containing yeast nutrients, salts, glucose, and 2 μg/ml tetracycline. Additional glucose was added during growth and at OD 550=20, indoleacrylic (IAA), a trp derepressor, was added to a concentration of 50 μg/ml. The cells were fed additional glucose to a final OD 550=40, achieved approximately 6 hours from the IAA addition.

E. coli (W3110) cells transformed with pKCEA trp 207-1* and double transformed (with pKCEAtrp207-1*Δ and pγCEAInt2) were grown in a manner analogous to that described above except that the OD 550 six hours after IAA addition at harvest was 25–30.

The cells were then harvested by centrifugation, and frozen.

E.2 Assay Method for Reconstituted Antibody

Anti-CEA activity was determined by ELISA as a criterion for successful reconstruction. Wells of microtiter plates (Dynatech Immulon) were saturated with CEA by incubation 100 μl of 2–5 μg CEA/ml solution in 0.1M carbonate buffer, pH 9.3 for 12 hours at room temperature. The wells were then washed 4 times with phosphate buffered saline (PBS), and then saturated with BSA by incubating 200 μl of 0.5 percent BSA in PBS for 2 hours at 37° C., followed by washing 4 times with PBS. Fifty microliters of each sample was applied to each well. A standard curve (shown in FIG. **10**), was run, which consisted of 50 μl samples of 10 μg, 5 μg, 1 μg, 500 ng, 100 ng, 50 ng, 10 ng, 5 ng and 1 ng anti-CEA/ml in 0.5 percent BSA in PBS, plus 50 μl of 0.5 percent BSA in PBS alone as a blank. All of the samples were incubated in the plate for 90 minutes at 37° C.

The plates were then washed 4 times with PBS, and sheep anti-mouse IgG-alkaline phosphate (TAGO, Inc.) was applied to each well by adding 100 μl of enzyme concentration of 24 units/ml in 0.5 percent BSA in PBS. The solution was incubated at 37° C. for 90 minutes. The plates wer washed 4 times with PBS before adding the substrate, 100 μl of a 0.4 mg/ml solution of p-nitrophenylphosphate (Sigma) in ethanolamine buffered saline, pH 9.5. The substrate was incubated 90 minutes at 37° C. for color development.

The A_{450} of each well was read by the Microelisa Auto Reader (Dynatech) set to a threshold of 1.5, calibration of 1.0 and the 0.5 percent BSA in PBS (Blank) well set to 0.000. The A_{450} data was tabulated in RS-1 on the VAX system, and the standard curve data fitted to a four-parameter logistic model. The unknown samples' concentrations were calculated based on the A_{450} data.

E.3 Reconstruction of Recombinant Antibody and Assay

Frozen cells prepared as described in paragraph E.1.10 were thawed in cold lysis buffer [10 mM Tris HCl, pH 7.5, 1 mM EDTA, 0.1M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and lysed by sonication. The lysate was partially clarified by centrifugation for 20 mins at 3,000 rpm. The supernatant was protected from proteolytic enzymes by an additional 1 mM PmSF, and used immediately or stored frozen at −80° C.; frozen lysates were never thawed more than once.

The S-sulfonate of *E. coli* produced anti-CEA heavy chain (γ) was prepared as follows: Recombinant *E. coli* cells transformed with pγCEAtrp207-1* which contained heavy chain as insoluble bodies, were lysed and centrifuged as above; the pellet was resuspended in the same buffer, sonicated and re-centrifuged. This pellet was washed once with buffer, then suspended in 6M guanidine HCl, 0.1M Tris HCl, pH 8, 1 mM EDTA, 20 mg/ml sodium sulfite and 10 mg/ml sodium tetrathionate and allowed to react at 25° for about 16 hrs. The reaction mixture was dialyzed against 8M urea, 0.1M Tris HCl, pH 8, and stored at 4°, to give a 3 mg/ml solution of γ-SSO_3.

650 μl of cell lysate from cells of various *E. coli* strains producing various IgG chains, was added to 500 mg urea. To this was added β-mercaptoethanol to 20 mM, Tris-HCl, pH 8.5 to 50 mM and EDTA to 1 mM, and in some experiments, γ-SSO_3 was added to 0.1 mg/ml. After standing at 25° for 30–90 mins., the reaction mixtures were dialyzed at 4° against a buffer com-

posed of 0.1M sodium glycinate, pH 10.8, 0.5M urea, 10 mM glycine ethyl ester, 5 mM reduced glutathione, 0.1 mM oxidized glutathione. This buffer was prepared from N_2-saturated water and the dialysis was performed in a capped Wheaton bottle. After 16–48 hours, dialysis bags were transferred to 4° phosphate buffered saline containing 1 mM PMSF and dialysis continued another 16–24 hrs. Dialysates were assayed by ELISA as described in paragraph E.2 for ability to bind CEA. The results below show the values obtained by comparison with the standard curve in x ng/ml anti-CEA. Also shown are the reconstruction efficiencies calculated from the ELISA responses, minus the background (108 ng/ml) of cells producing K chain only, and from estimates of the levels of γ and K chains in the reaction mixtures.

	ng/ml anti-CEA	Percent recombination
E. coli W3110 producing IFN-αA (control)	0	—
E. coli (W3110/pKCEAtrp207-1*)	108	—
E. coli (W3110/pKCEAtrp207-1*), plus γ-SSO_3	848	0.33
E. coli (W3110/pKCEAtrp207-1*Δ, pγCEA Int2)	1580	0.76
Hybridoma anti-CEA K-SSO_3 and γ-SSO_3	540	0.40

E.4 Preparation of Chimeric Antibody

FIGS. 11 and 12 show the construction of an expression vector for a chimeric heavy (gamma) chain which comprises the murine anti CEA variable region and human γ-2 constant region.

A DNA sequence encoding the human gamma-2 heavy chain is prepared as follows: the cDNA libary obtained by standard techniques from a human multiple myeloma cell line is probed with 5' GGGCACT-CGACACAA 3' to obtain the plasmid containing the cDNA insert for human gamma-2 chain (Takahashi, et al., *Cell*, 29: 671 (1982), incorporated herein by reference), and analyzed to verify its identity with the known sequence in human gamma-2 (Ellison, J., et al., *Proc. Natl. Acad. Sci. (USA)*, 79: 1984 (1982) incorporated herein by reference).

As shown in FIG. 11, two fragments are obtained from this cloned human gamma 2 plasmid (pγ2). The first fragment is formed by digestion with PvuII followed by digestion with Ava III, and purification of the smaller DNA fragment, which contains a portion of the constant region, using 6 percent PAGE. The second fragment is obtained by digesting the pγ2 with any restriction enzyme which cleaves in the 3' untranslated region of γ2, as deduced fromthe nucleotide sequence, filling in with Klenow and dNTPs, cleaving with Ava III, and isolating the smaller fragment using 6 percent PAGE. (The choice of a two step, two fragment composition to supply the PvuII-3' untranslated fragment provides a cleaner path to product due to the proximity of the AVAIII site to the 3 terminal end thus avoiding additional restriction sites in the gene sequence matching the 3' untranslated region site.) pγCEA207-1* is digested with EcoR 1, treated with Klenow and dNTPs to fill in the cohesive end, and digested with Pvu II, the large vector fragment containing promoter isolated by 6 percent PAGE.

The location and DNA sequence surrounding the PvuII site in the mouse gamma-1 gene are identical to the location and DNA sequence surrounding the PvuII site in the human gamma-2 gene.

The plasmid resulting from a three way ligation of the foregoing fragments, pChim1, contains, under the influence of trp promoter, the variable and part of the constant region of murine anti-CEA gamma 1 chain, and a portion of the gamma 2 human chain. pChim1 will, in fact, express a chimeric heavy chain when tranformed into *E. coli*, but one wherein the change from mouse to human does not take place at the variable to constant junction.

FIG. 12 shows modification of pChim1 to construct pChim2 so that the resulting protein from expression will contain variable region from murine anti CEA antibody and constant region from the human γ-2 chain. First, a fragment is prepared from pChim1 by treating with Nco I, blunt ending with Klenow and dNTPs, cleaving with Pvu II, and isolating the large vector fragment which is almost the complete plasmid except for short segment in the constant coding region for mouse anti CEA. A second fragment is prepared from the previously described pγ2 by treating with Pvu II, followed by treating with any restriction enzyme which cleaves in the variable region, blunt ending with Klenow and dNTPs and isolating the short fragment which comprises the junction between variable and constant regions of this chain.

Ligation of the foregoing two fragments produces an intermediate plasmid which is correct except for an extraneous DNA fragment which contains a small portion of the constant region of the murine anti CEA antigen, and a small portion of the variable region of the human gamma chain. This repair can be made by excising the Xba I to Pvu II fragment and cloning into M13 phage as described by Messing et al., *Nucleic Acids Res.* 9: 309 (1981), followed by in vitro site directed deletion mutagenesis as described by Adelman, et al., DNA, in press (1983) which is incorporated herein by reference. The Xba I-Pvu II fragment thus modified is ligated back into the intermediate plasmid to form pChim2. this plasmid then is capable of expressing in a suitable host a cleanly constructed murine variable/human constant chimeric heavy chain.

In an analogous fashion, but using mRNA templates for cDNA construction for human kappa rather than γ chain, the expression plasmid for chimeric light chain is prepared.

The foregoing two plasmids are then double transformed into *E. coli* W3110, the cells grown and the chains reconstituted as set forth in paragraph E.1–E.3 supra.

E.5 Preparation of Altered Murine Anti-CEA Antibody

E.5.1 Construction of Plasmid Vectors for Direct Expression of Altered Murine Anti-CEA Heavy Chain Gene

The cysteine residues, and the resultant disulfide bonds in the region of amino acids 216–230 in the constant region of murine anti-CEA heavy chain are suspected to be imported for complement fixation (Klein, et al., *Proc. Natl. Acad. Sci., (USA)*, 78: 524 (1981)) but not for the antigen binding property of the resulting antibody. To decrease the probability of incorrect disulfide bond formation during reconstruction according to the process of the invention herein, the nucleotides encoding the amino acid residues 226–232 which includes codons for three cysteines, are deleted as follows:

A "deleter" deoxyoligonucelotide, 5' CTAACAC-CATGTCAGGGT is used to delete the relevant portions of the gene from pγCEAtrp207-1* by the procedure of Wallace, et al., *Science*, 209: 1396 (1980). Briefly, the "deleter" deoxyoligonucelotide is annealed with denatured pγCEAtrp207-1* DNA, and primer repair synthesis carried out in vitro, followed by screening by hybridization of presumptive deletion clones with P^{32} labelled deleter sequence.

E.5.2 Production of Cysteine Deficient Altered Antibody

The plasmid prepared in E.5.1 is transformed into an *E.coli* strain previously transformed with pKCEAtrp207-1* as described above. The cells are grown, extracted for recombinant antibody chains, and the altered antibody reconstituted as described in E.1.10.

E.6 Preparation of Fab

E.6.1 Construction of a Plasmid Vector for Direct Expression of Murine Anti-CEA Gamma 1 Fab Fragment Gene pγCEAFabtrp207-1*

FIG. 13 presents the construction of pγCEA-Fabtrp207-1*. 5 μg of pBR322 was digested with Hind III, the cohesive ends made flush by treating with Klenow and dNTPs; digested with Pst I, and treated with BAP. The large vector fragment, fragment I, was recovered using 6 percent PAGE followed by electroelution.

5 μg of pγCEAtrp207-1* was digested with both BamH I and Pst I and the ~1570 bp DNA fragment (fragment II) containing the trp promoter and the gene sequence encoding the variable region continuing into constant region and further into the anti-CEA gamma 1 chain hinge region, was isolated and purified after electrophoresis.

Expression of the anti-CEA gamma 1 chain Fab fragment rather than complete heavy chain requires that a termination condon be constructed at the appropriate location in the gene. For this, the 260 bp Nco I-Nde I DNA fragment from 20 μg of the pγ298 was isolated and purified. A 13 nucleotide DNA primer, the complement of which encodes the last 3 C-terminal amino acids of the Fab gene and 2 bases of the 3 needed for the stop codon, was synthesized by the phosphotriester method (supra). The probe hybridizes to nucleotides 754 to 767 (FIG. 4) which has the following sequence:

Asp Cys Gly Stop
5' GGGATTGTGGTTG 3'

The third base of the stop codon is provided by the terminal nucleotide of the filled-in Hind III site from pBR322 cleavage described above. 500 ng of this primer was used in a primer repair reaction by phosphorylation at the 5' end in a reaction with 10 units T4 DNA kinase containing 0.5 mM ATP in 20 μl, and mixing with ~200 ng of the Nco I-Nde I DNA fragment. The mixture was heat denatured for 3 minutes at 95° and quenched in dry-ice ethanol. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C., this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, digested with BamH I and the reaction electrophoresed through a 6 percent polyacrylamide gel. ~50 ng of the 181 bp blunt end to BamH I DNA fragment, fragment III, was isolated and purified.

~100 ng of fragment I, ~100 ng each of fragments II and III were ligated overnight and transformed into *E. coli* K12 strain 294. Plasmid DNA from several tetracycline resistant transformants was analyzed for the proper construction and the nucleotide sequence through the repair blunt end filled-in Hind III junction was determined for verification of the TGA stop codon.

E.6.2 Production of Fab Protein

The plasmid prepared in E.6.1 is transformed into an *E. coli* strain previously transformed with pKCEAtrp207-1* as described above. The cells are grown, extracted for recombinant antibody chains and the Fab protein reconstituted as described in E.1.10.

We claim:

1. A method comprising
   (a) preparing a DNA sequence encoding a chimeric immunoglobulin heavy or light chain having specificity for a particular known antigen wherein a constant region is homologous to the corresponding constant region of an antibody of a first mammalian species and a variable region thereof is homologous to the variable region of an antibody derived from a second, different mammalian species;
   (b) inserting the sequence into a replicable expression vector operably linked to a suitable promoter compatible with a host cell;
   (c) transforming the host cell with the vector of (b);
   (d) culturing the host cell; and
   (e) recovering the chimeric heavy or light chain from the host cell culture.

2. The method of claim 1 wherein the first mammalian species is human.

3. A composition comprising a chimeric immunoglobulin heavy or light chain having specificity for a particular known antigen having a constant region homologous to a corresponding constant region of an antibody of a first mammalian species and a variable region homologous to a variable region of an antibody derived from a second, different mammalian species.

4. The chimeric heavy or light chain of clain 3 wherein the constant region is human.

5. A replicable expression vector comprising DNA operably linked to a promoter compatible with a suitable host cell, said DNA encoding a chimeric immunoglobulin heavy or light chain having specificity for a particular known antigen and having a constant region homologous to a corresponding region of an antibody of a first mammalian species and a variable region homologous to a variable region of an antibody derived from a second, different mammalian species.

6. The vector of claim 5 wherein the first mammalian species is human.

7. Recombinant host cells transformed with the vector of claim 5.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,816,567 Page 1 of 3

DATED : March 28, 1989

INVENTOR(S) : Shmuel Cabilly et al.

It is certified that error appears in the above - identified patent and that said Letters Patent is hereby corrected as shown below:

On the title page, left col. line 10, insert in addition to "Assignee: Genentech, Inc.", --City of Hope, Duarte, Calif.--.

On Col. 5, line 10, change "immunoglobuins" to --immunoglobulins--.

On Col. 1, line 17, insert --,-- after "proteins".

On Col. 4, line 9, change "reach" to --reached--.

On Col. 5, line 14, delete ".".

On Col. 6, line 28, delete the second "antibodies".

On col. 6, line 32, change "reference" to --referenced--.

On Col. 7, line 8, change "respones" to --responses--.

On Col. 9, line 7, change "F^{32}" to --F^{-}--.

On Col. 10, line 21, change "Examlples" to --Examples--.

On Col. 16, line 1, change "particular" to --particularly--.

On Col. 16, line 54, change "formaion" to --formation--.

On Col. 17, line 38, change "liner" to --linear--.

On Col. 17, line 62, change "for" to --from--.

On Col. 18, line 38, change "mous" to --mouse--.

On Col. 19, line 27, change "from" to --for--.

On Col. 20, line 57, change "207-1," to --207-1.--.

On Col. 21, line 6, change "(pH 7.4)" to --(pH 7.4),--.

On Col. 21, line 21, change "isolted" to --isolated--.

On Col. 21, line 23, change "precipitaion" to --precipitation--.

On Col. 21, line 23, change "resuspensionin" to --resuspension in--.

On Col. 21, line 58, change "sequene" to --sequence--.

On Col. 22, line 5, change "isoated" to --isolated--.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,816,567

DATED : March 28, 1989

INVENTOR(S) : Shmuel Cabilly et al.

It is certified that error appears in the above - identified patent and that said Letters Patent is hereby corrected as shown below:

On Col. 22, line 19, change "reactin" to --reaction--.

On Col. 22, line 25, change "digested" to --designated--.

On Col. 22, line 30, change "PvaII" to --PvuII--.

On Col. 22, line 54, change "regin" to --region--.

On Col. 23, line 44, change "ahd" to --and--.

On Col. 23, line 55, change "μreaction" to --μl reaction--.

On Col. 24, line 9, change "blund" to --blunt--.

On Col. 25, line 16, change "tranformed" to --transformed--.

On Col. 25, line 47, change "preparation" to --preparations--.

On Col. 25, line 55, change "transformed" to --transferred--.

On Col. 26, line 5, change "reconstruction" to --reconstitution--.

On Col. 26, line 7, change "incubation" to --incubating--.

On Col. 26, line 25, change "wer" to --were--.

On Col. 27, line 12, change "reconstruction" to --reconstitution--.

On Col. 27, line 37, change "myleoma" to --myeloma--.

On Col. 27, line 60, change "AVAIII" to --AvaIII--.

On Col. 28, line 64, change "reconstruction" to --reconstitution--.

On Col. 29, line 38, change "condon" to --codon--.

On Col. 30, line 48, change "clain" to --claim--.

On Col. 30, line 55, insert --constant-- between "corresponding" and "region".

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 4,816,567 Page 3 of 3

DATED : March 28, 1989

INVENTOR(S) : Shmuel Cabilly, et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On Col. 21, line 58, change "sequene" to --sequence--.

On Col. 22, line 5, change "isoated" to --isolated--.

Signed and Sealed this

Fourth Day of August, 1992

Attest:

DOUGLAS B. COMER

Attesting Officer

Acting Commissioner of Patents and Trademarks

US006331415B1

(12) **United States Patent**
Cabilly et al.

(10) **Patent No.: US 6,331,415 B1**
(45) **Date of Patent: Dec. 18, 2001**

(54) **METHODS OF PRODUCING IMMUNOGLOBULINS, VECTORS AND TRANSFORMED HOST CELLS FOR USE THEREIN**

(75) Inventors: **Shmuel Cabilly**, Monrovia; **Herbert L. Heyneker**, Burlingame; **William E. Holmes**, Pacifica; **Arthur D. Riggs**, La Verne; **Ronald B. Wetzel**, San Francisco, all of CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **07/205,419**

(22) Filed: **Jun. 10, 1988**

Related U.S. Application Data

(63) Continuation of application No. 06/483,457, filed on Apr. 8, 1983, now Pat. No. 4,816,567.

(51) **Int. Cl.**[7] **C12N 15/13**; C12N 15/00; C12N 15/63

(52) **U.S. Cl.** **435/69.6**; 435/69.1; 435/69.7; 435/70.1; 435/70.21; 435/71.1; 435/71.2; 435/320; 435/252.1; 435/252.3; 435/252.33; 435/254.11; 435/254.2; 435/254.21; 435/455; 435/471; 435/483; 435/485

(58) **Field of Search** 435/69.1, 69.7, 435/71.1, 70.1, 71.2, 320, 261, 252.1, 252.3, 81, 55, 56, 69.6, 252.33, 254.21, 483

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Primary Examiner—Phillip Gambel
(74) *Attorney, Agent, or Firm*—Burns, Doane, Swecker & Mathis, LLP

(57) **ABSTRACT**

The invention relates to processes for producing an immunoglobulin or an immunologically functional immunoglobulin fragment containing at least the variable domains of the immunoglobulin heavy and light chains. The processes can use one or more vectors which produce both the heavy and light chains or fragments thereof in a single cell. The invention also relates to the vectors used to produce the immunoglobulin or fragment, and to cells transformed with the vectors.

36 Claims, 19 Drawing Sheets

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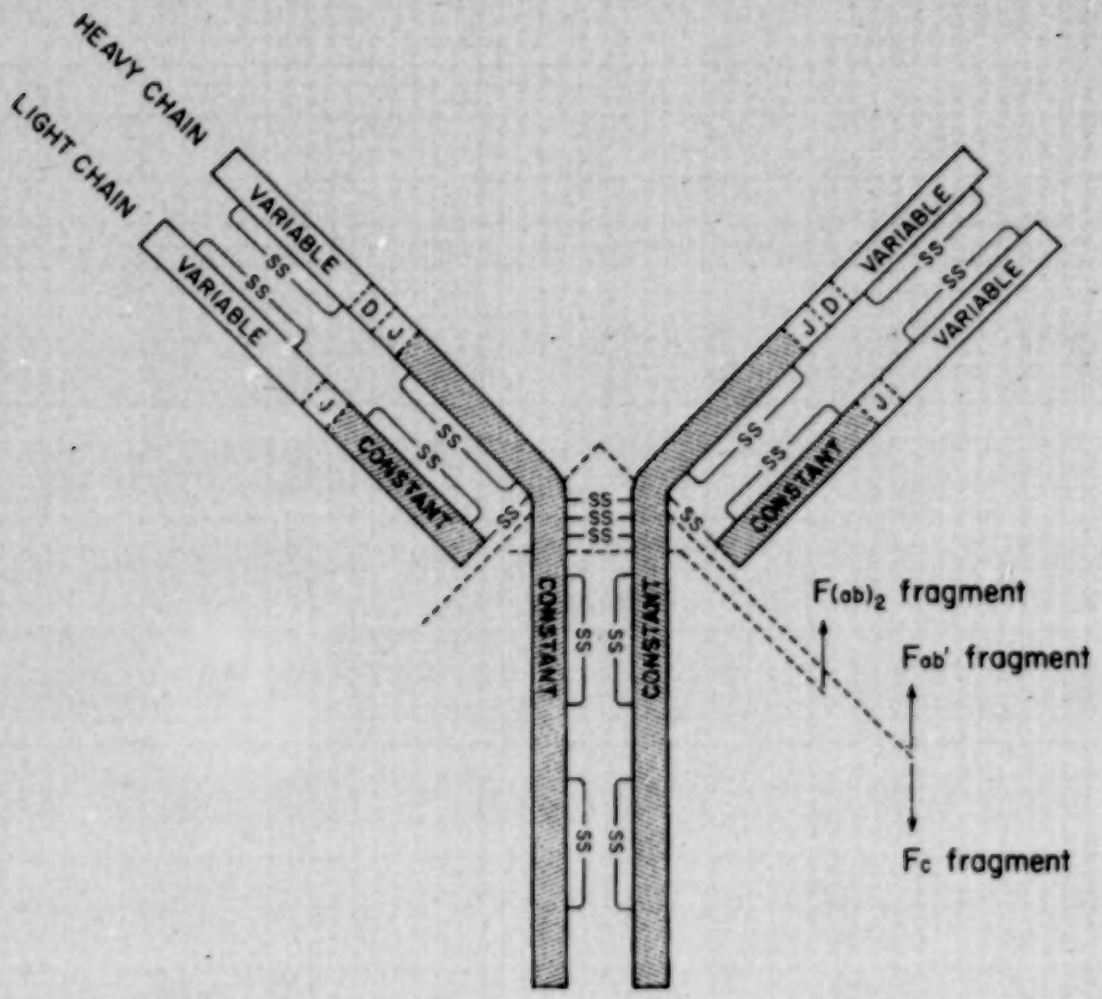

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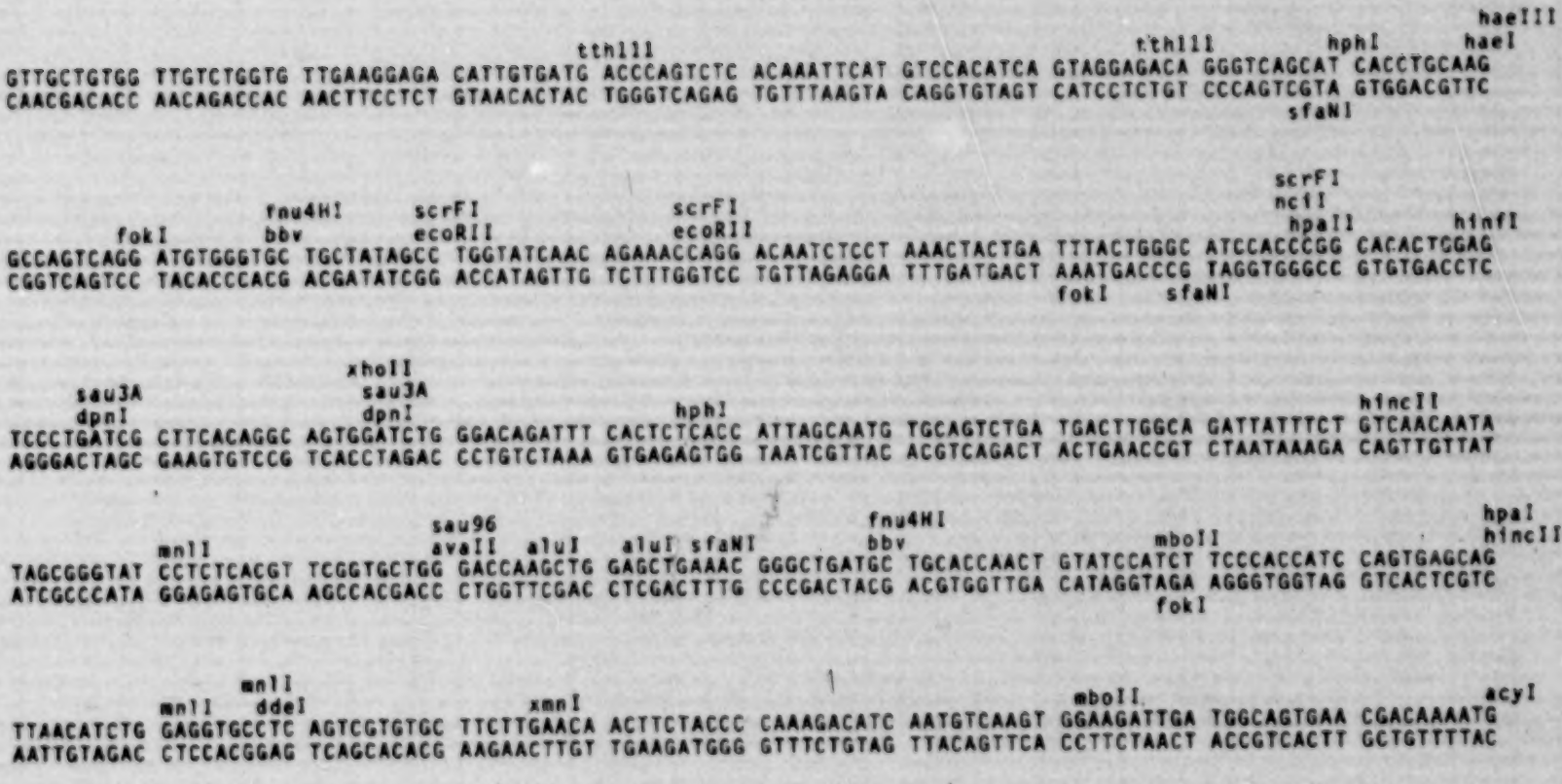
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Fig. 2A.

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                          dpnI                                        fnu4HI
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                mnlI                                                                                  sau96   hgaI
                  haeIII                                                                                ddeI
                 haeI                          hphI            aluI                                   avaII   acyI
601   CTATACCTGT GAGGCCACTC ACAAGACATC AACTTCACCC ATTGTCAAGA GCTTCAACAG GAATGAGTGT TAGAGACAAA GGTCCTGAGA CGCCACCACC
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                                                                                                        mnlI
      aluI    aluI          mboII    ddeI          mnlI                                  hgiA          mnlI          mnlI
701   AGCTCCCCAG CTCCATCCTA TCTTCCCTTC TAAGGTCTTG GAGGCTTCCC CACAAGCGAC CTACCACTGT TGCGGTGCTC CAAACCTCCT CCCCACCTCC
      TCGAGGGGTC GAGGTAGGAT AGAAGGGAAG ATTCCAGAAC CTCCGAAGGG GTGTTCGCTG GATGGTGACA ACGCCACGAG GTTTGGAGGA GGGGTGGAGG
          fokI

            mnlI
        mnlI   mnlI                                       xmnI                hinfI
801   TTCTCCTCCT CCTCCCTTTC CTTGGCTTTT ATCATGCTAA TATTTGCAGA AAATATTCAA TAAAGTGAGT CTTTGCACTT GA
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Fig. 2B.

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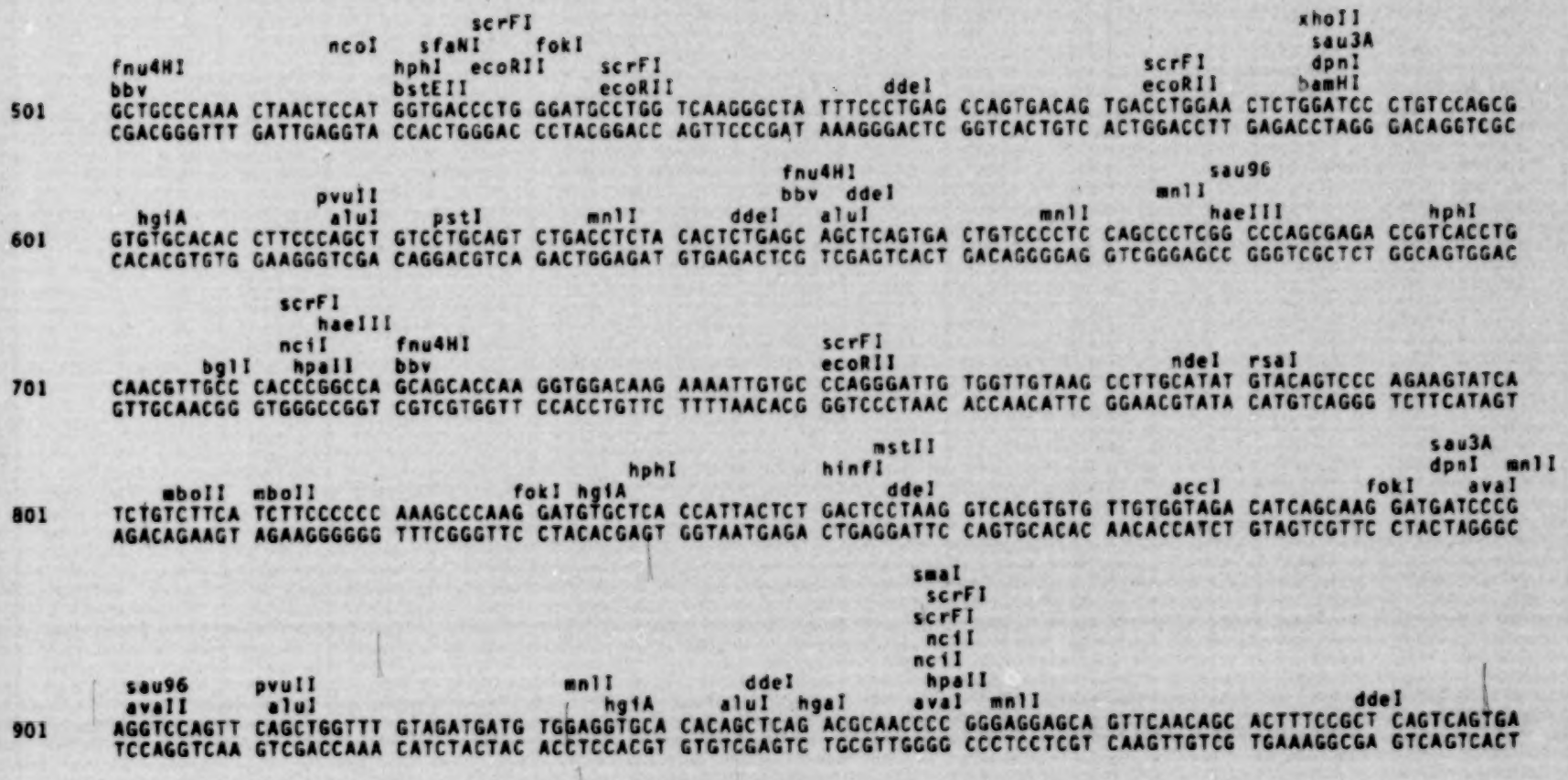
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scrFI
haeIII
nciI fnu4HI scrFI
bglI hpaII bbv ecoRII ndeI rsaI
701 CAACGTTGCC CACCCGGCCA GCAGCACCAA GGTGGACAAG AAAATTGTGC CCAGGGATTG TGGTTGTAAG CCTTGCATAT GTACAGTCCC AGAAGTATCA
GTTGCAACGG GTGGGCCGGT CGTCGTGGTT CCACCTGTTC TTTTAACACG GGTCCCTAAC ACCAACATTC GGAACGTATA CATGTCAGGG TCTTCATAGT
mstII
sau3A
hphI hinfI dpnI mnlI
mboII mboII fokI hgiA ddeI accI fokI aval
801 TCTGTCTTCA TCTTCCCCCC AAAGCCCAAG GATGTGCTCA CCATTACTCT GACTCCTAAG GTCACGTGTG TTGTGGTAGA CATCAGCAAG GATGATCCCG
AGACAGAAGT AGAAGGGGGG TTTCGGGTTC CTACACGAGT GGTAATGAGA CTGAGGATTC CAGTGCACAC AACACCATCT GTAGTCGTTC CTACTAGGGC
smaI
scrFI
scrFI
nciI
nciI
hpaII
sau96 pvuII mnlI ddeI
avaII aluI hgiA aluI hgaI aval mnlI ddeI
901 AGGTCCAGTT CAGCTGGTTT GTAGATGATG TGGAGGTGCA CACAGCTCAG ACGCAACCCC GGGAGGAGCA GTTCAACAGC ACTTTCCGCT CAGTCAGTGA
TCCAGGTCAA GTCGACCAAA CATCTACTAC ACCTCCACGT GTGTCGAGTC TGCGTTGGGG CCCTCCTCGT CAAGTTGTCG TGAAAGGCGA GTCAGTCACT

Fig. 4B.

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```

```
                                                                  250
ile phe pro pro lys pro lys asp val leu thr ile thr leu thr pro lys val thr cys val val val asp ile ser lys asp asp pro
AUC UUC CCC CCA AAG CCC AAG GAU GUG CUC ACC AUU ACU CUG ACU CCU AAG GUC ACG UGU GUU GUG GUA GAC AUC AGC AAG GAU GAU CCC

                                                                  280
glu val gln phe ser trp phe val asp asp val glu val his thr ala gln thr gln pro arg glu glu gln phe asn ser thr phe arg
GAG GUC CAG UUC AGC UGG UUU GUA GAU GAU GUG GAG GUG CAC ACA GCU CAG ACG CAA CCC CGG GAG GAG CAG UUC AAC AGC ACU UUC CGC

                                                                  310
ser val ser glu leu pro ile met his gln asp trp leu asn gly lys glu phe lys cys arg val asn ser ala ala phe pro ala pro
UCA GUC AGU GAA CUU CCC AUC AUG CAC CAG GAC UGG CUC AAU GGC AAG GAG UUC AAA UGC AGG GUC AAC AGU GCA GCU UUC CCU GCC CCC

                                                                  340
ile glu lys thr ile ser lys thr lys gly arg pro lys ala pro gln val tyr thr ile pro pro pro lys glu gln met ala lys asp
AUC GAG AAA ACC AUC UCC AAA ACC AAA GGC AGA CCG AAG GCU CCA CAG GUG UAC ACC AUU CCA CCU CCC AAG GAG CAG AUG GCC AAG GAU

                                                                  370
lys val ser leu thr cys met ile thr asp phe phe pro glu asp ile thr val glu trp gln trp asn gly gln pro ala glu asn tyr
AAA GUC AGU CUG ACC UGC AUG AUA ACA GAC UUC UUC CCU GAA GAC AUU ACU GUG GAG UGG CAG UGG AAU GGG CAG CCA GCG GAG AAC UAC

                                                                  400
lys asn thr gln pro ile met asn thr asn gly ser tyr phe val tyr ser lys leu asn val gln lys ser asn trp glu ala gly asn
AAG AAC ACU CAG CCC AUC AUG AAC ACG AAU GGC UCU UAC UUC GUC UAC AGC AAG CUC AAU GUG CAG AAG AGC AAC UGG GAG GCA GGA AAU

                                                                  430
thr phe thr cys ser val leu his glu gly leu his asn his his thr glu lys ser leu ser his ser pro gly lys OP
ACU UUC ACC UGC UCU GUG UUA CAU GAG GGC CUG CAC AAC CAC CAU ACU GAG AAG AGC CUC UCC CAC UCU CCU GGU AAA UGA UCCCAGUGUCCU

UGGAGCCCUCUGGUCCUACAGGACUCUGACACCUACCUCCACCCCUCCCUGUAUAAAUAAAGCACCCAGCACUGCCCUUGGGAAAAA
```

Fig. 5B.

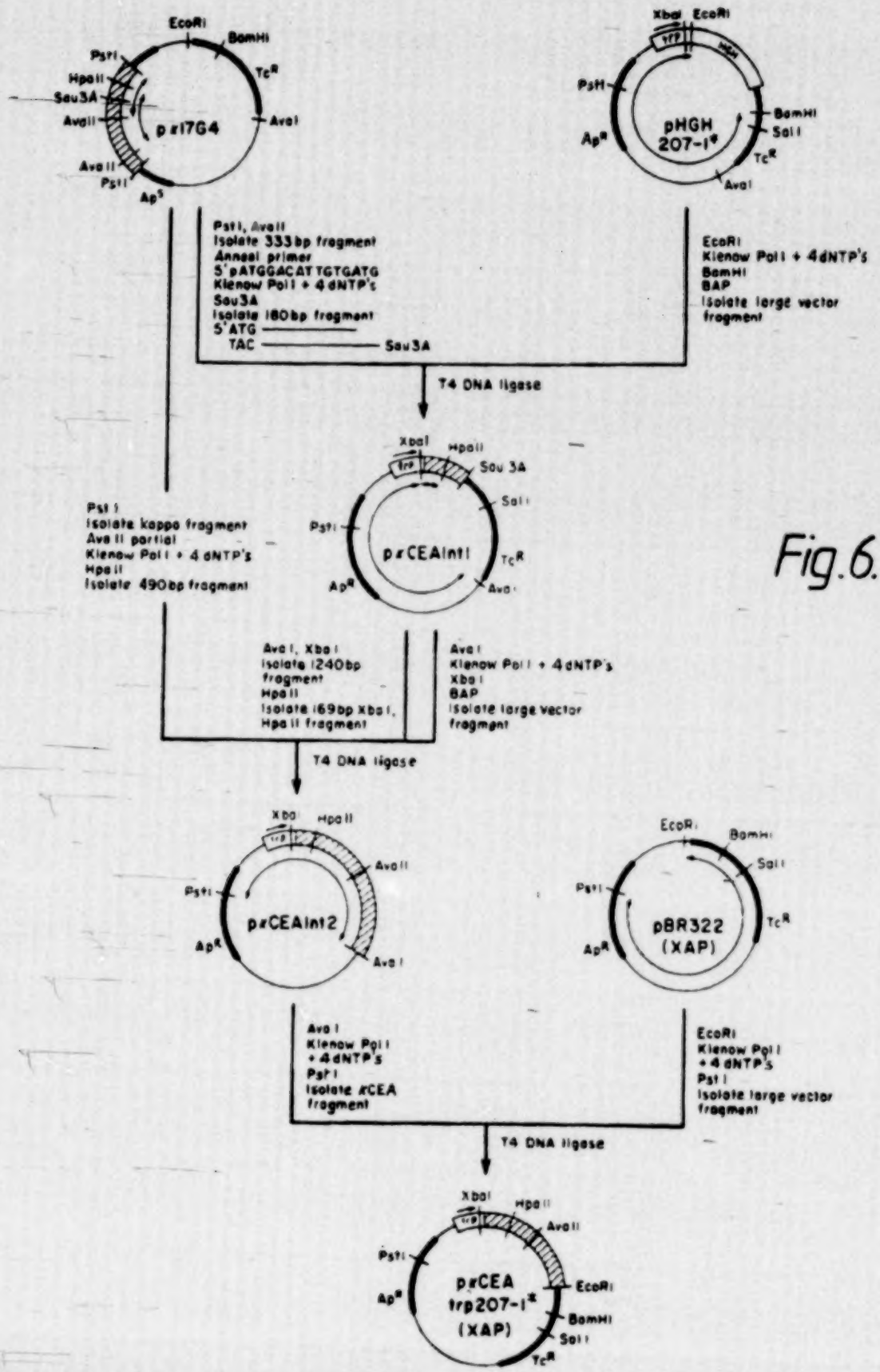
EcoRI
BamHI
PstI
HpaII
Sau3A
AvaII
TcR
AvaI
pαI7G4
ApS
PstI, AvaII
Isolate 333bp fragment
Anneal primer
5' pATGGACATTGTGATG
Klenow PolI + 4 dNTP's
Sau3A
Isolate 180bp fragment
5' ATG
TAC
Sau3A
XbaI EcoRI
trp
PsH
pHGH
207-1*
ApR
BamHI
SalI
TcR
AvaI
EcoRI
Klenow PolI + 4dNTP's
BamHI
BAP
Isolate large vector fragment
T4 DNA ligase
XbaI
HpaII
Sau 3A
SalI
PstI
pαCEAint1
TcR
ApR
AvaI
PstI
Isolate kappa fragment
AvaII partial
Klenow PolI + 4 dNTP's
HpaII
Isolate 490bp fragment
AvaI, XbaI
Isolate 1240bp fragment
HpaII
Isolate 169bp XbaI, HpaII fragment
AvaI
Klenow PolI + 4dNTP's
XbaI
BAP
Isolate large vector fragment
T4 DNA ligase
XbaI
HpaII
AvaII
PstI
pαCEAint2
ApR
AvaI
EcoRI
BamHI
SalI
PstI
pBR322
(XAP)
TcR
ApR
AvaI
Klenow PolI + 4dNTP's
PstI
Isolate αCEA fragment
EcoRI
Klenow PolI + 4 dNTP's
PstI
Isolate large vector fragment
T4 DNA ligase
XbaI
HpaII
AvaII
PstI
pαCEA
trp207-1*
(XAP)
ApR
EcoRI
BamHI
SalI
TcR

Fig.6.

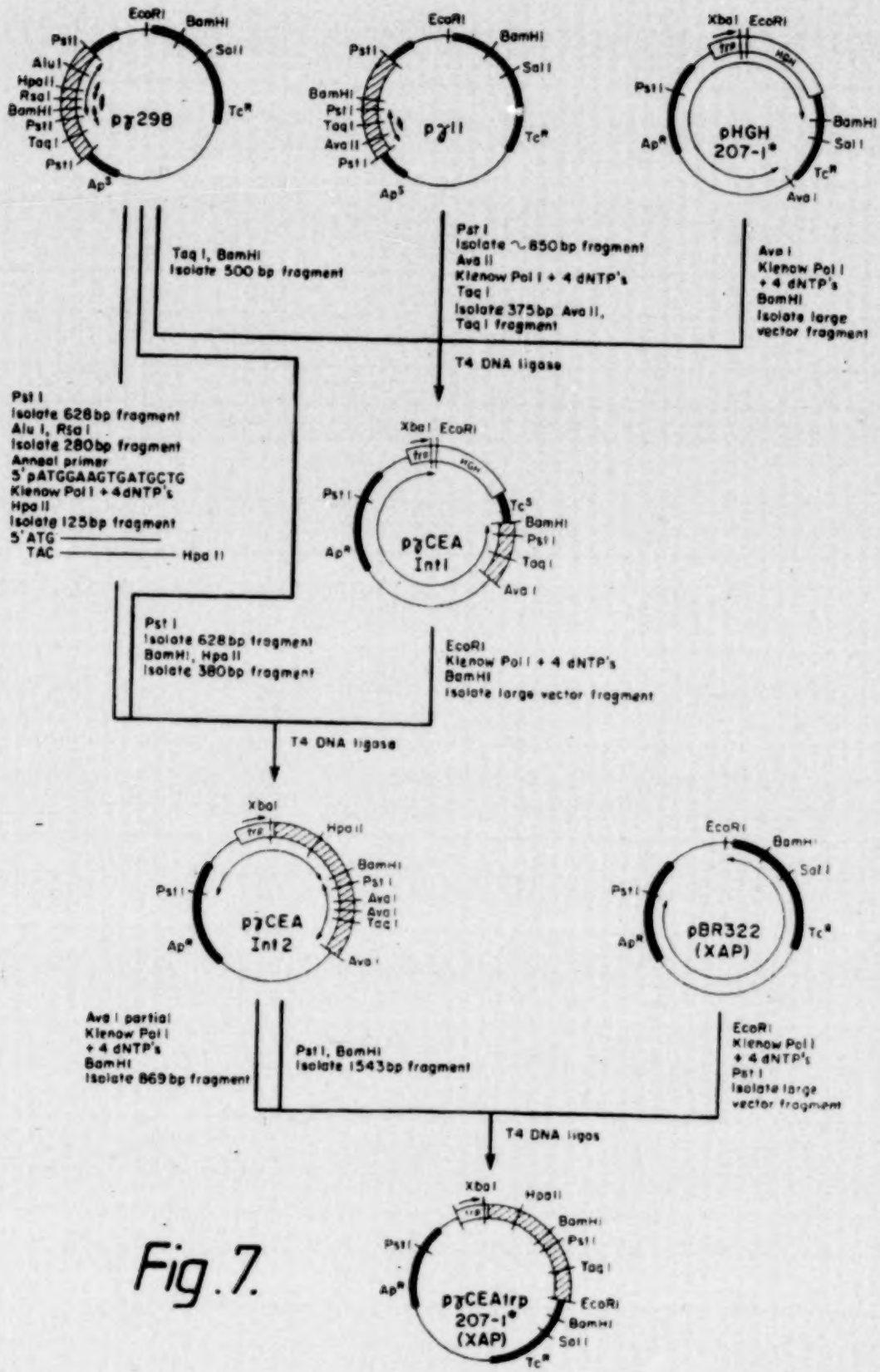
EcoRI
BamHI
Sal I
Tc^R
pγ298
Pst I
Alu I
Hpa II
Rsa I
BamHI
Pst I
Taq I
Pst I
Ap^S
pγ11
Ava II
pHGH 207-1*
Xba I
trp
HGH
Ap^R
Ava I
Taq I, BamHI
Isolate 500 bp fragment
Pst I
Isolate ~850 bp fragment
Ava II
Klenow Pol I + 4 dNTP's
Taq I
Isolate 375 bp Ava II, Taq I fragment
Ava I
Klenow Pol I + 4 dNTP's
BamHI
Isolate large vector fragment
T4 DNA ligase
Pst I
Isolate 628 bp fragment
Alu I, Rsa I
Isolate 280 bp fragment
Anneal primer
5' pATGGAAGTGATGCTG
Klenow Pol I + 4 dNTP's
Hpa II
Isolate 125 bp fragment
5' ATG
TAC
Hpa II
pγCEA Int1
Tc^S
Pst I
Taq I
Ava I
Pst I
Isolate 628 bp fragment
BamHI, Hpa II
Isolate 380 bp fragment
EcoRI
Klenow Pol I + 4 dNTP's
BamHI
Isolate large vector fragment
T4 DNA ligase
Xba I
Hpa II
pγCEA Int2
Ava I
Ava I
Taq I
pBR322 (XAP)
Ava I partial
Klenow Pol I
+ 4 dNTP's
BamHI
Isolate 869 bp fragment
Pst I, BamHI
Isolate 1543 bp fragment
EcoRI
Klenow Pol I
+ 4 dNTP's
Pst I
Isolate large vector fragment
T4 DNA ligas
pγCEAIrp 207-1* (XAP)

Fig. 7.

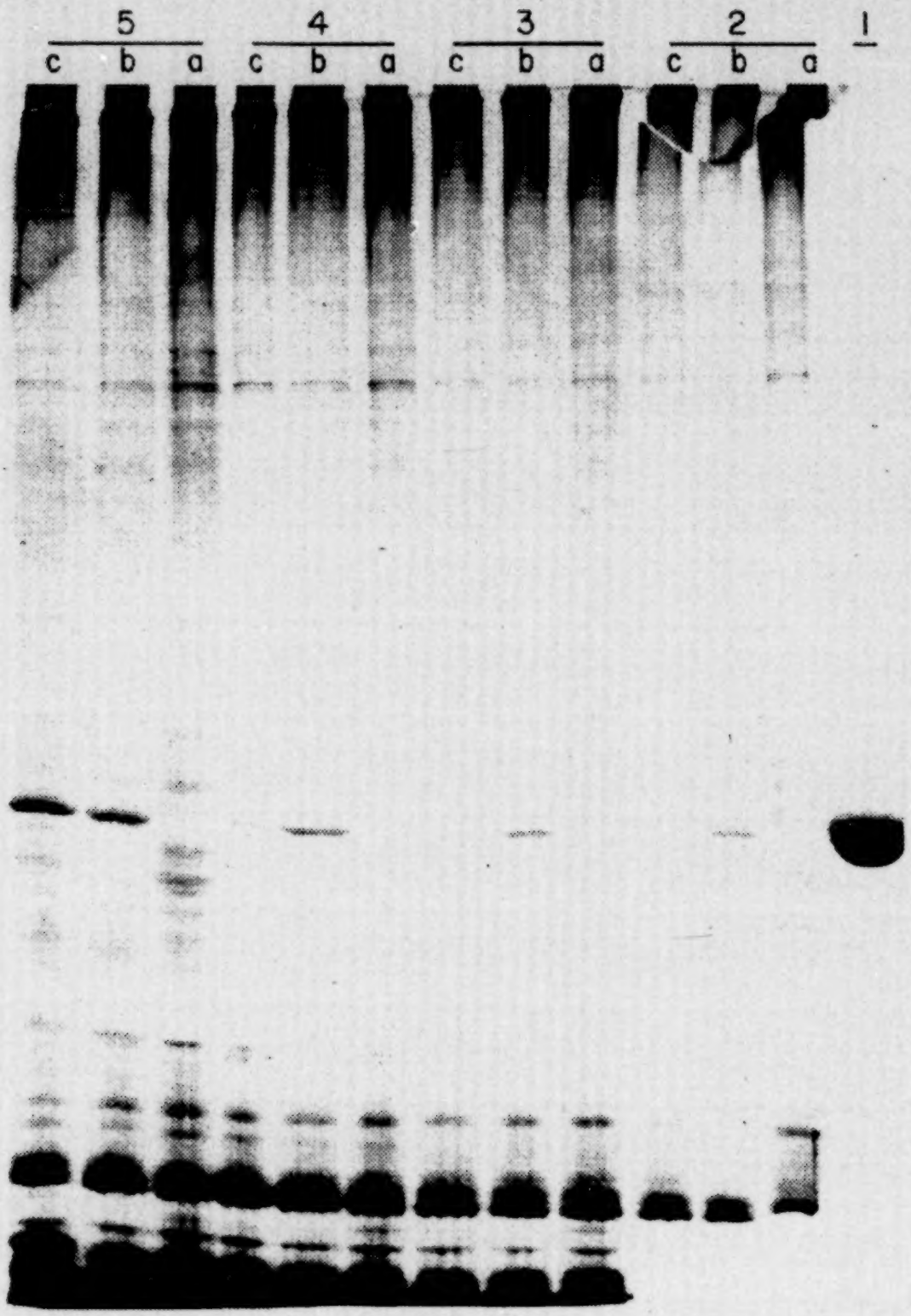

Fig. 8A.

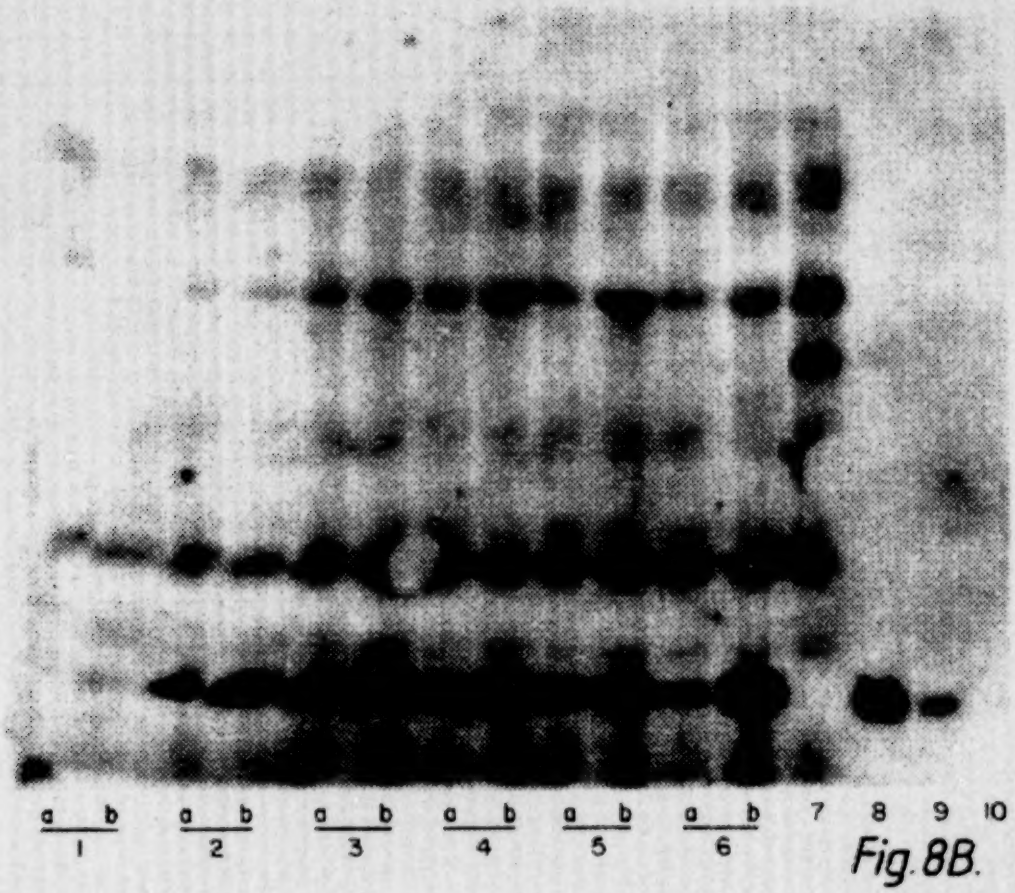

Fig. 8B.

Fig. 8C.

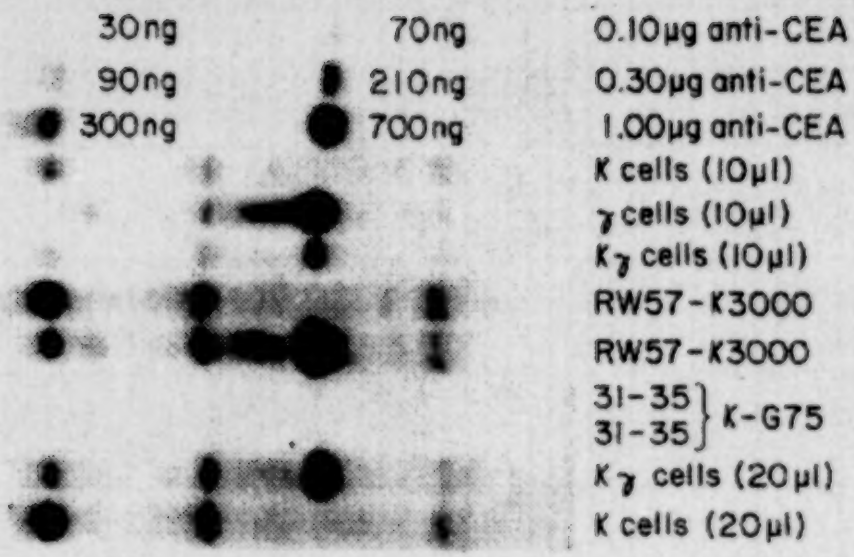
30ng
70ng
0.10μg anti-CEA
90ng
210ng
0.30μg anti-CEA
300ng
700ng
1.00μg anti-CEA
K cells (10μl)
γ cells (10μl)
Kγ cells (10μl)
RW57-K3000
RW57-K3000
31-35
31-35
K-G75
Kγ cells (20μl)
K cells (20μl)

Fig. 9.

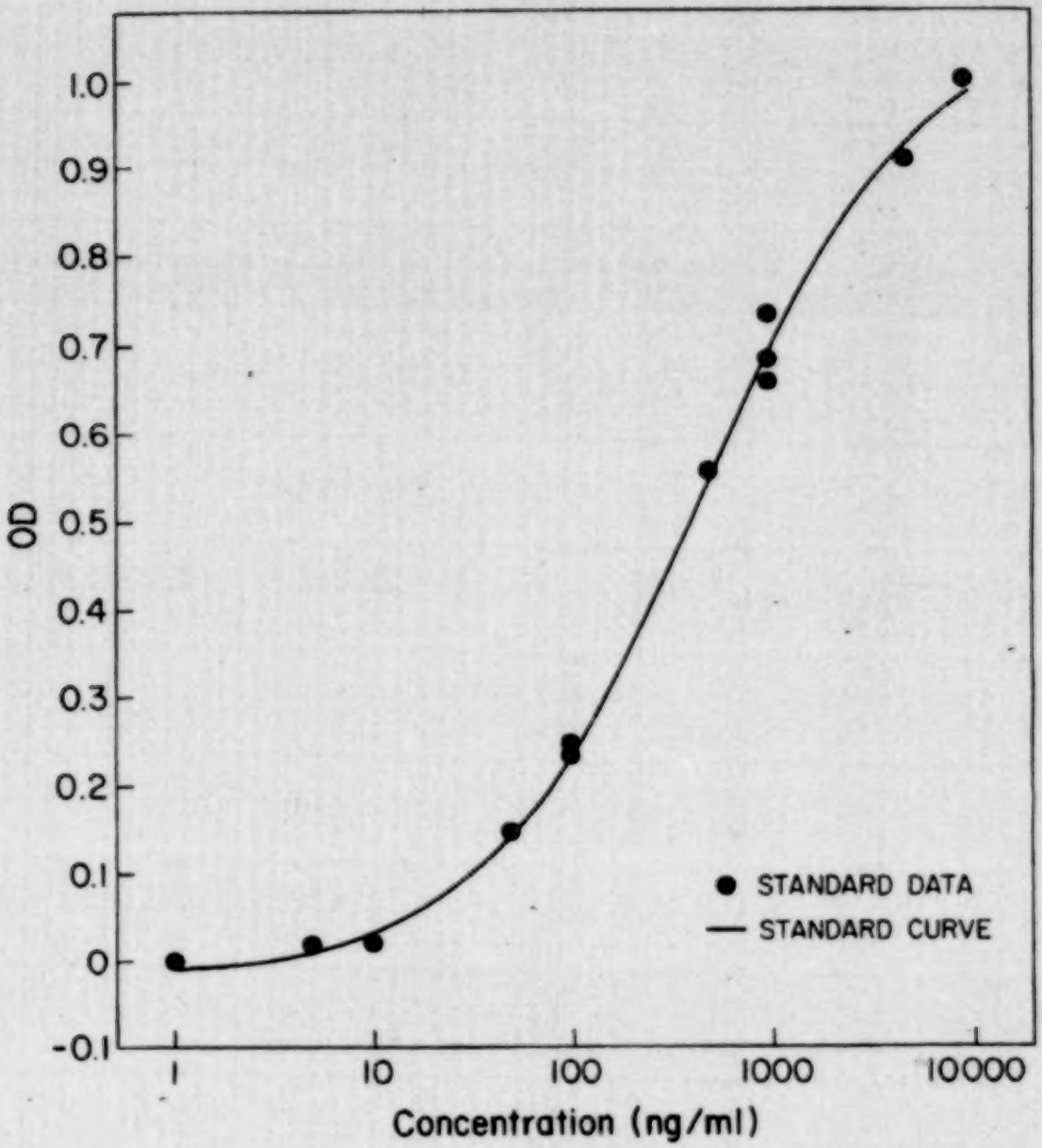
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
OD
1
10
100
1000
10000
Concentration (ng/ml)
STANDARD DATA
STANDARD CURVE

Fig. 10.

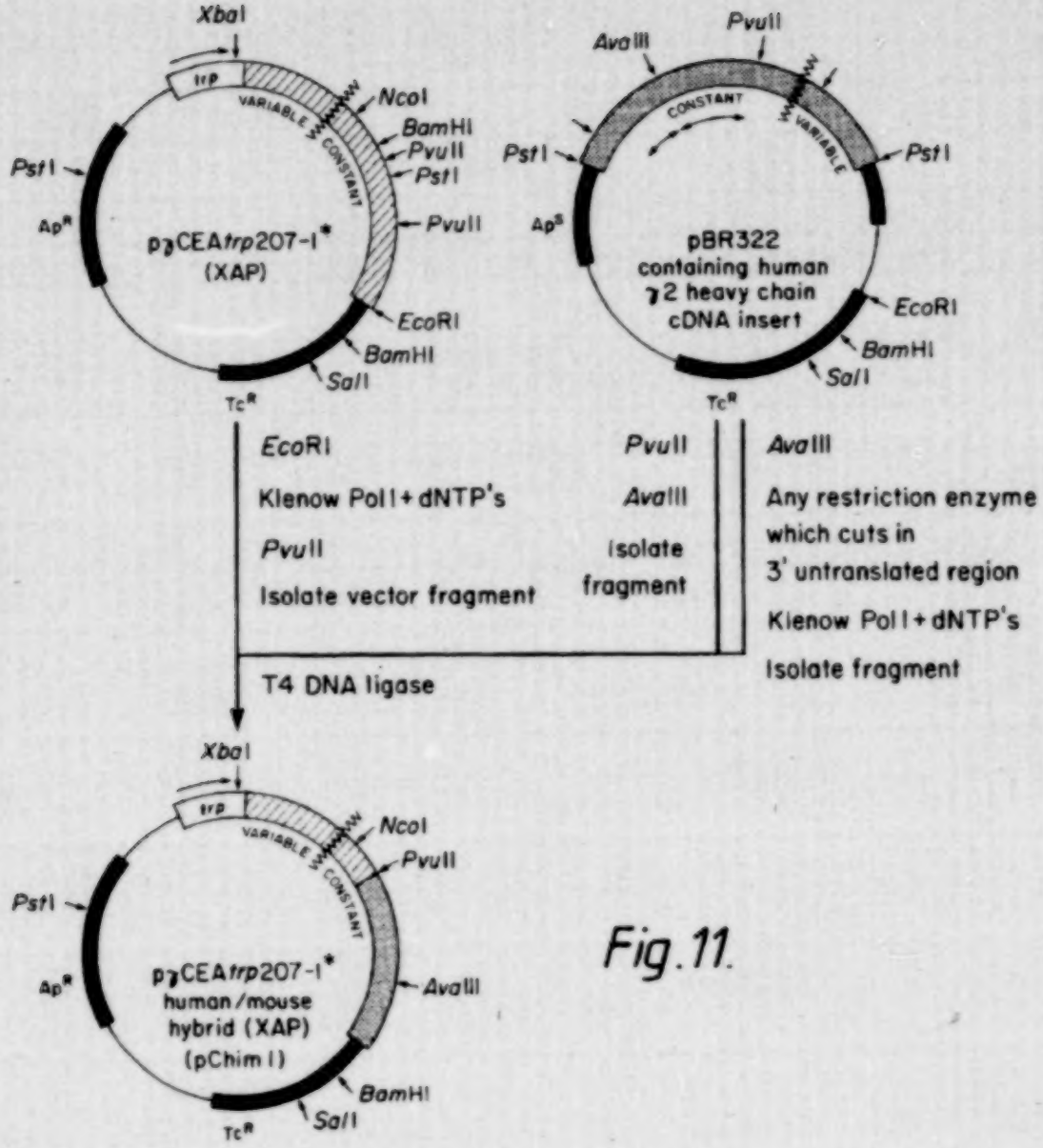
XbaI
trp
VARIABLE
CONSTANT
NcoI
BamHI
PvuII
PstI
PvuII
EcoRI
BamHI
SalI
PstI
Ap^R
pγCEAtrp207-1*
(XAP)
Tc^R
AvaIII
PvuII
CONSTANT
VARIABLE
PstI
PstI
Ap^S
pBR322
containing human
γ2 heavy chain
cDNA insert
EcoRI
BamHI
SalI
Tc^R
EcoRI
Klenow PolI + dNTP's
PvuII
Isolate vector fragment
PvuII
AvaIII
Isolate
fragment
AvaIII
Any restriction enzyme
which cuts in
3' untranslated region
Klenow PolI + dNTP's
Isolate fragment
T4 DNA ligase
XbaI
trp
VARIABLE
CONSTANT
NcoI
PvuII
PstI
AvaIII
Ap^R
pγCEAtrp207-1*
human/mouse
hybrid (XAP)
(pChim I)
BamHI
SalI
Tc^R

Fig. 11.

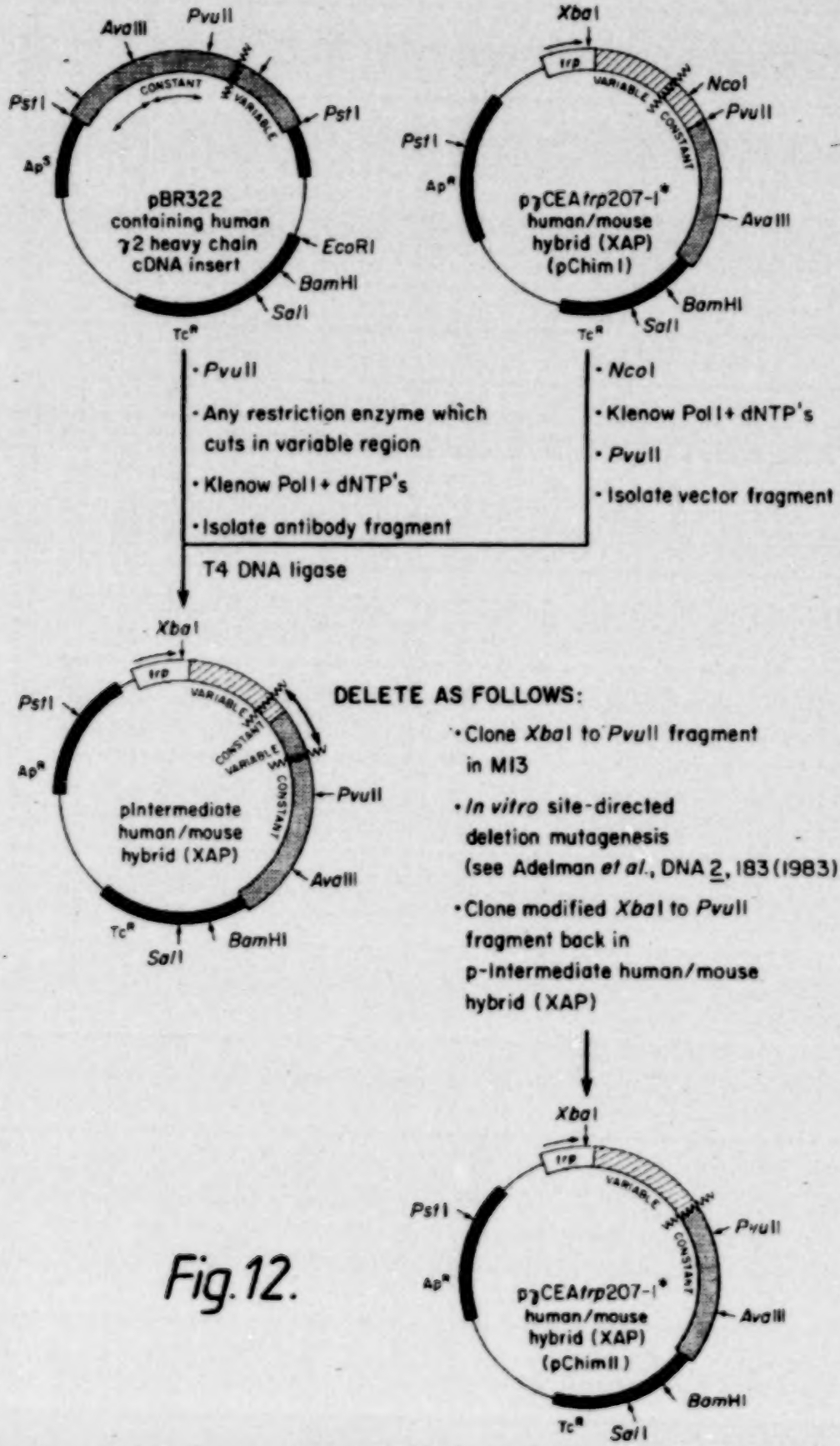
AvaIII
PvuII
PstI
PstI
Ap^R
CONSTANT
VARIABLE
pBR322
containing human
γ2 heavy chain
cDNA insert
EcoRI
BamHI
SalI
Tc^R
XbaI
trp
VARIABLE
CONSTANT
NcoI
PvuII
PstI
Ap^R
pγCEAtrp207-1*
human/mouse
hybrid (XAP)
(pChim1)
AvaIII
BamHI
SalI
Tc^R
• PvuII
• Any restriction enzyme which cuts in variable region
• Klenow Pol I + dNTP's
• Isolate antibody fragment
• NcoI
• Klenow Pol I + dNTP's
• PvuII
• Isolate vector fragment
T4 DNA ligase
XbaI
trp
VARIABLE
CONSTANT
VARIABLE
CONSTANT
PstI
Ap^R
PvuII
pIntermediate
human/mouse
hybrid (XAP)
AvaIII
Tc^R
SalI
BamHI
DELETE AS FOLLOWS:
• Clone XbaI to PvuII fragment in M13
• In vitro site-directed deletion mutagenesis (see Adelman et al., DNA 2, 183 (1983))
• Clone modified XbaI to PvuII fragment back in p-Intermediate human/mouse hybrid (XAP)
XbaI
trp
VARIABLE
CONSTANT
PstI
PvuII
Ap^R
pγCEAtrp207-1*
human/mouse
hybrid (XAP)
(pChim11)
AvaIII
BamHI
Tc^R
SalI

Fig. 12.

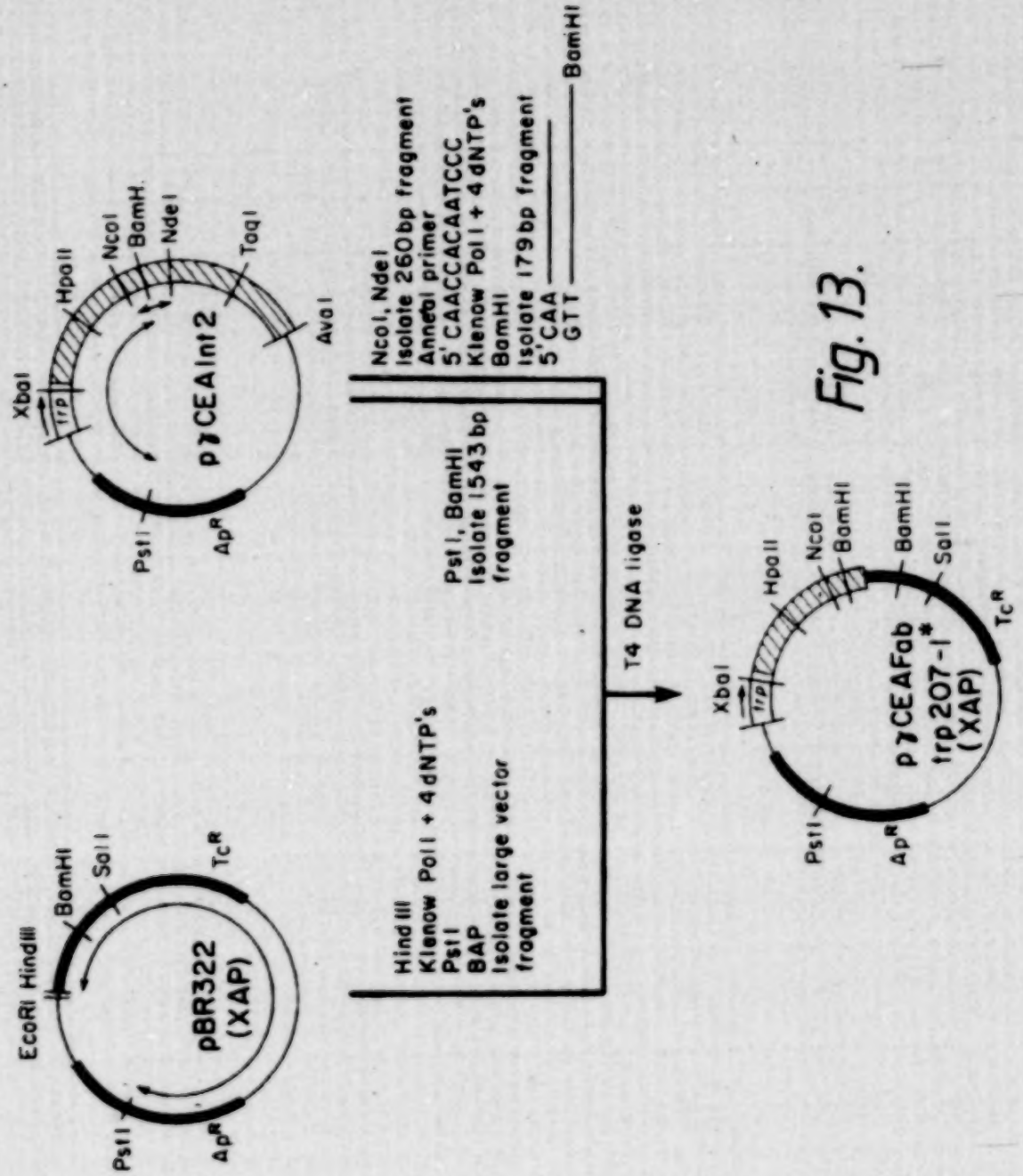
EcoRI HindIII
BamHI
SalI
TcR
pBR322
(XAP)
PstI
ApR
XbaI
trp
HpaII
NcoI
BamH.
NdeI
TaqI
AvaI
p7CEAInt2
PstI
ApR
HindIII
Klenow PolI + 4 dNTP's
PstI
BAP
Isolate large vector fragment
PstI, BamHI
Isolate 1543 bp fragment
NcoI, NdeI
Isolate 260bp fragment
Anneal primer
5' CAACCACAATCCC
Klenow PolI + 4 dNTP's
BamHI
Isolate 179 bp fragment
5'CAA
GTT
BamHI
T4 DNA ligase
XbaI
trp
HpaII
NcoI
BamHI
BamHI
SalI
TcR
p7CEAFab
trp207-1*
(XAP)
PstI
ApR

Fig. 13.

METHODS OF PRODUCING IMMUNOGLOBULINS, VECTORS AND TRANSFORMED HOST CELLS FOR USE THEREIN

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 06/483,457, filed Apr. 8, 1983, now U.S. Pat. No. 4,816,567, issued Mar. 28, 1989.

BACKGROUND OF THE INVENTION

This invention relates to the field of immunoglobulin production and to modification of naturally occurring immunoglobulin amino acid sequences. Specifically, the invention relates to using recombinant techniques to produce both immunoglobulins which are analogous to those normally found in vertebrate systems and to take advantage of these gene modification techniques to construct chimeric or other modified forms.

A. Immunoglobulins and Antibodies

Antibodies are specific immunoglobulin polypeptides produced by the vertebrate immune system in response to challenge by foreign proteins, glycoproteins, cells, or other antigenic foreign substances. The sequence of events which permits the organism to overcome invasion by foreign cells or to rid the system of foreign substances is at least partially understood. An important part of this process is the manufacture of antibodies which bind specifically to a particular foreign substance. The binding specificity of such polypeptides to a particular antigen is highly refined, and the multitude of specificities capable of being generated by the individual vertebrate is remarkable in its complexity and variability. Thousands of antigens are capable of eliciting responses, each almost exclusively directed to the particular antigen which elicited it.

Immunoglobulins include both antibodies, as above described, and analogous protein substances which lack antigen specificity. The latter are produced at low levels by the lymph system and in increased levels by myelomas.

A.1 Source and Utility

Two major sources of vertebrate antibodies are presently utilized—generation in situ by the mammalian B lymphocytes and in cell culture by B-cell hybrids. Antibodies are made in situ as a result of the differentiation of immature B lymphocytes into plasma cells, which occurs in response to stimulation by specific antigens. In the undifferentiated B cell, the portions of DNA coding for the various regions on the immunoglobulin chains are separated in the genomic DNA. The sequences are reassembled sequentially prior to transcription. A review of this process has been given by Gough, *Trends in Biochem Sci*, 6: 203 (1981). The resulting rearranged genome is capable of expression in the mature B lymphocyte to produce the desired antibody. Even when only a single antigen is introduced into the sphere of the immune system for a particular mammal, however, a uniform population of antibodies does not result. The in situ immune response to any particular antigen is defined by the mosaic of responses to the various determinants which are present on the antigen. Each subset of homologous antibody is contributed by a single population of B-cells—hence in situ generation of antibodies is "polyclonal".

This limited but inherent heterogeneity has been overcome in numerous particular cases by use of hybridoma technology to create "monoclonal" antibodies (Kohler, et al., *Eur J. Immunol*., 6: 511 (1976)). In this process, splenocytes

or lymphocytes from a mammal which has been injected with antigen are fused with a tumor cell line, thus producing hybrid cells or "hybridomas" which are both immortal and capable of producing the genetically coded antibody of the B cell. The hybrids thus formed we segregated into single genetic strains by selection, dilution, and regrowth, and each strain thus represents a single genetic line. They therefore produce immunoreactive antibodies against a desired antigen which are assured to be homogenous, and which antibodies, referencing their pure genetic parentage, are called "monoclonal". Hybridoma technology has to this time been focused largely on the fusion of murine lines, but human-human hybridomas (Olsson, L. et al., *Proc. Natl. Acad. Sci. (USA)*, 77: 5429 (1980)); human-murine hybridomas (Schlom, J., et al. (ibid) 77: 6841 (1980)) and several other xenogenic hybrid combinations have been prepared as well. Alternatively, primary, antibody producing, B cells have been immortalized in vitro by transformation with viral DNA.

Polyclonal, or, much more preferably monoclonal, antibodies have a variety of useful properties similar to those of the present invention. For example, they can be used as specific immunoprecipitating reagents to detect the presence of the antigen which elicited the initial processing of the B cell genome by coupling this antigen-antibody reaction with suitable detection techniques such as labeling with radioisotopes or with enzymes capable of assay (RIA, EMIT, and ELISA). Antibodies are thus the foundation of immuno diagnostic tests for many antigenic substances. In another important use, antibodies can be directly injected into subjects suffering from an attack by a substance or organism containing the antigen in question to combat this attack. This process is currently in its experimental stages, but its potential is clearly seen. Third, whole body diagnosis and treatment is made possible because injected antibodies are directed to specific target disease tissues, and thus can be used either to determine the presence of the disease by carrying with them a suitable label, or to attack the diseased tissue by carrying a suitable drug.

Monoclonal antibodies produced by hybridomas, while theoretically effective as suggested above and clearly preferable to polyclonal antibodies because of their specificity, suffer from certain disadvantages. First, they tend to be contaminated with other proteins and cellular materials of hybridoma, (and, therefore, mammalian) origin. These cells contain additional materials, notably nucleic acid fragments, but protein fragments as well, which are capable of enhancing, causing, or mediating carcinogic responses. Second, hybridoma lines producing monoclonal antibodies tend to be unstable and may alter the structure of antibody produced or stop producing antibody altogether (Kohler, G., et al. *Proc. Natl. Acad. Sci (USA)* 77: 2197 (1980); Morrison, S. L., *J. Immunol*. 123: 793 (1979)). The cell line genome appears to alter itself in response to stimuli whose nature is not currently known, and this alteration may result in production of incorrect sequences. Third, both hybridoma and B cells inevitably produce certain antibodies in glycosylated form (Melchers, F., *Biochemistry*, 10: 653 (1971)) which, under some circumstances, may be undesirable. Fourth, production of both monoclonal and polyclonal antibodies is relatively expensive. Fifth, and perhaps most important, production by current techniques (either by hybridoma or by B cell response) does not permit manipulation of the genome so as to produce antibodies with more effective design components than those normally elicited in response to antigens from the mature B cell in situ. The antibodies of the present invention do not suffer from the

foregoing drawbacks, and, furthermore, offer the opportunity to provide molecules of superior design.

Even those immunoglobulins which lack the specificity of antibodies are useful, although over a smaller spectrum of potential uses than the antibodies themselves. In presently understood applications, such immunoglobulins are helpful in proteins replacement therapy for globulin related anemia. In this context, an inability to bind to antigen is in fact helpful, as the therapeutic value of these proteins would be impaired by such functionality. At present, such non-specific antibodies are derivable in quantity only from myeloma cell cultures suitably induced. The present invention offers an alternative, more economical source. It also offers the opportunity of canceling out specificity by manipulating the four chains of the tetramer separately.

A.2 General Structure Characteristics

The basic immunoglobin structural unit in vertebrate systems is now well understood (Edelman, G. M., *Ann. N.Y. Acad. Sci.*, 190: 5 (1971)). The units are composed of two identical light polypeptide chains of molecular weight approximately 23,000 daltons, and two identical heavy chains of molecular weight 53,000–70,000. The four chains are joined by disulfide bonds in a "Y" configuration wherein the light chains bracket-the heavy-chains starting at the mouth of the Y and continuing through the divergent region as shown in FIG. 1. The "branch" portion, as there indicated, is designated the Fab region. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, with some subclasses among them, and the nature of this chain, as it has a long constant region, determines the "class"of the antibody as IgG, IgM, IgA, IgD, or IgE. Light chains are classified-as either kappa or lambda. Each heavy chain class can be prepared with either kappa or lambda light chain. The light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages when the immunoglobulins are generated either by hybridomas or by B cells. However, if non-covalent association of the chains can be effected in the correct geometry, the aggregate will still be capable of reaction with antigen, or of utility as a protein supplement as a non-specific immunoglobulin.

The amino acid sequence runs from the N-terminal end at the top of the Y to the C-terminal end at the bottom of each chain. At the N-terminal end is a variable region which is specific for the antigen which elicited it, and is approximately 100 amino acids in length, there being slight variations between light and heavy chain and from antibody to antibody. The variable region is linked in each chain to a constant region which extends the remaining length of the chain. Linkage is seen, at the genomic level, as occurring through a linking sequence known currently as the "J" region in the light chain gene, which encodes about 12 amino acids, and as a combination of "D" region and "J" region in the heavy chain gene, which together encode approximately 25 amino acids.

The remaining portions of the chain are referred to as constant regions and within a particular class do not to vary with the specificity of the antibody (i.e., the antigen eliciting it).

As stated above, there are five known major classes of constant regions which determine the class of the immunoglobulin molecule (IgG, IgM, IgA, IgD, and IgE corresponding to γ, μ, α, δ, and ϵ heavy chain constant regions). The constant region or class determines subsequent effector function of the antibody, including activation of complement (Kabat, E. A., *Structural Concepts in Immunology and Immunochemistry*, 2nd Ed., p. 413–436, Holt, Rinehart Winston (1976)), and other cellular responses (Andrews, D. W., et al., *Clinical Immunobiology* pp 1–18, W. B. Sanders (1980); Kohl, S., et al., *Immunology*, 48: 187 (1983)); while the variable region determines the antigen with which it will react.

B. Recombinant DNA Technology

Recombinant DNA technology has reached sufficient sophistication that it includes a repertoire of techniques for cloning and expression of gene sequences. Various DNA sequences can be recombined with some facility, creating new DNA entities capable of producing heterologous protein product in transformed microbes and cell cultures. The general means and methods for the in vitro ligation of various blunt ended or "sticky" ended fragments of DNA, for producing expression vectors, and for transforming organisms are now in hand.

DNA recombination of the essential elements (i.e., an origin of replication, one or more phenotypic selection characteristics, expression control sequence, heterologous gene insert and remainder vector) generally is performed outside the host cell. The resulting recombinant replicable expression vector, or plasmid, is introduced into cells by transformation and large quantities of the recombinant vehicle is obtained by growing the transformants. Where the gene is properly inserted with reference to portions which govern the transcription and translation of the encoded DNA message, the resulting expression vector is useful to produce the polypeptide sequence for which the inserted gene codes, a process referred to as "expression." The resulting product may be obtained by lysis, if necessary, of the host cell and recovery of the product by appropriate purifications from other proteins.

In practice, the use of recombinant DNA technology can express entirely heterologous polypeptides—so-called direct expression—or alternatively may express a heterologous polypeptide fused to a portion of the amino acid sequence of a homologous polypeptide. In the latter cases, the intended bioactive product is sometimes rendered bioinactive within the fused, homologous/heterologous polypeptide until it is cleaved in an extracellular environment.

The art of maintaining cell or tissue cultures as well as microbial systems for studying genetics and cell physiology is well established. Means and methods are available for maintaining permanent cell lines, prepared by successive serial transfers from isolated cells. For use in research, such cell lines are maintained on a solid support in liquid medium, or by growth in suspension containing support nutriments. Scale-up for large preparations seems to pose only mechanical problems.

SUMMARY OF THE INVENTION

The invention relates to antibodies and to non-specific immunoglobulins (NSIs) formed by recombinant techniques using suitable host cell cultures. These antibodies and NSIs can be readily prepared in pure "monoclonal" form. They can be manipulated at the genomic level to produce chimeras of variants which draw their homology from species which differ from each other. They can also be manipulated at the protein level, since all four chains do not need to be produced by the same cell. Thus, there are a number of "types" of immunoglobulins encompassed by the invention.

First, immunoglobulins, particularly antibodies, are produced using recombinant techniques which mimic the amino acid sequence of naturally occurring antibodies produced by either mammalian B cells in situ, or by B cells fused with suitable immortalizing tumor lines, i.e., hybridomas.

Second, the methods of this invention produce, and the invention is directed to, immunoglobulins which comprise polypeptides not hitherto found associated with each other in nature. Such reassembly is particularly useful in producing "hybrid" antibodies capable of binding more than one antigen; and in producing "composite" immunoglobulins wherein heavy and light chains of different origins essentially damp out specificity. Third, by genetic manipulation, "chimeric" antibodies can be formed wherein, for example, the variable regions correspond to the amino acid sequence from one mammalian model system, whereas the constant region mimics the amino acid sequence of another. Again, the derivation of these two mimicked sequences may be from different species. Fourth, also by genetic manipulation, "altered" antibodies with improved specificity and other characteristics can be formed.

Two other types of immunoglobulin-like moieties may be produced: "univalent" antibodies, which are useful as homing carriers to target tissues, and "Fab proteins" which include only the "Fab" region of an immunoglobulin molecule i.e., the branches of the "Y". These univalent antibodies and Fab fragments may also be "mammalian" i.e., mimic mammalian amino acid sequences; novel assemblies of mammalian chains, or chimeric, where for example, the constant and variable sequence patterns may be of different origin. Finally, either the light chain or heavy chain alone, or portions, thereof, produced by recombinant techniques are included in the invention and may be mammalian or chimeric.

In other aspects, the invention is directed to DNA which encodes the aforementioned NSIs, antibodies, and portions thereof, as well as expression vectors or plasmids capable of effecting the production of such immunoglobulins in suitable host cells. It includes the host cells and cell cultures which result from transformation with these vectors. Finally, the invention is directed to methods of producing these NSIs and antibodies, and the DNA sequences, plasmids, and transformed cells intermediate to them.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a representation of the general structure of immunoglobulins.

FIGS. **2A–B** shows the detailed sequence of the cDNA insert of pK17G4 which encodes kappa anti CEA chain.

FIG. **3** shows the coding sequence of the fragment shown in FIG. **2**, along with the corresponding amino acid sequence.

FIGS. **4A–C** shows the combined detailed sequence of the cDNA inserts of pγ298 and pγ11 which encode gamma anti CEA chain.

FIGS. **5A–B** shows the corresponding amino acid sequence encoded by the fragment in FIG. **4**.

FIGS. **6** and **7** outline the construction of expression vectors for kappa and gamma anti-CEA chains respectively.

FIGS. **8A**, **8B**, and **8C** show the results of sizing gels run on, extracts of *E. coli* expressing the genes for gamma chain, kappa chain, and both kappa and gamma chains respectively.

FIG. **9** shows the results of western blots of extracts of cells transformed as those in FIGS. **8**.

FIG. **10** shows a standard curve for ELISA assay of anti CEA activity.

FIGS. **11** and **12** show the construction of a plasmid for expression of the gene encoding a chimeric heavy chain.

FIG. **13** shows the construction of a plasmid for expression of the gene encoding the Fab region of heavy chain.

DETAILED DESCRIPTION

A. Definitions

As used herein, "antibodies" refers to tetramers or aggregates thereof which have specific immunoreactive activity, comprising light and heavy chains usually aggregated in the "Y" configuration of FIG. **1**, with or without covalent linkage between them; "immunoglobulins" refers to such assemblies whether or not specific immunoreactive activity is a property. "Non-specific immunoglobulin" ("NSI") means those immunoglobulins which do not possess specificity—i.e., those which are not antibodies.

"Mammalian antibodies" refers to antibodies wherein the amino acid sequences of the chains are homologous with those sequences found in antibodies produced by mammalian systems, either in situ, or in hybridomas. These antibodies mimic antibodies which are otherwise capable of being generated, although in impure form, in these traditional systems.

"Hybrid antibodies" refers to antibodies wherein chains are separately homologous with referenced mammalian antibody chains and represent novel assemblies of them, so that two different antigens are precipitable by the tetramer. In hybrid antibodies, one pair of heavy and light chain is homologous to antibodies raised against one antigen, while the other pair of heavy and light chain is homologous to those raised against another antigen. This results in the property of "divalence" i.e., ability to bind two antigens simultaneously. Such hybrids may, of course, also be formed using chimeric chains, as set forth below.

"Composite" immunoglobulins means those wherein the heavy and light chains mimic those of different species origins or specificities, and the resultant is thus likely to be a non-specific immunoglobulin (NSI), i.e.—lacking in antibody character.

"Chimeric antibodies" refers to those antibodies wherein one portion of each of the amino acid sequences of heavy and light chains is homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular class, while the remaining segment of the chains is homologous to corresponding sequences in another. Typically, in these chimeric antibodies, the variable region of both light and heavy chains mimics the variable regions of antibodies derived from one species of mammals while the constant portions are homologous to the sequences in antibodies derived from another. One clear advantage to such chimeric forms is that, for example, the variable regions can conveniently be derived from presently known sources using readily available hybridomas or B cells from non human host organisms in combination with constant regions derived from, for example, human cell preparations. While the variable region has the advantage of ease of preparation, and the specificity is not affected by its source, the constant region being human, is less likely to elicit an immune response from a human subject when the antibodies are injected than would the constant region from a non-human source.

However, the definition is not limited to this particular example. It includes any antibody in which either or both of the heavy or light chains are composed of combinations of sequences mimicking the sequences in antibodies of different sources, whether these sources be differing classes, differing antigen responses, or differing species of origin and whether or not the fusion point is At the variable constant boundary. Thus, it is possible to produce antibodies in which neither the constant nor the variable region mimic known antibody sequences. It then becomes possible, for example, to construct antibodies whose variable region has a higher

specific affinity for a particular antigen, or whose constant region can elicit enhanced complement fixation or to make other improvements in properties possessed by a particular constant region.

"Altered antibodies" means antibodies wherein the amino acid sequence has been varied from that of a mammalian or other vertebrate antibody. Because of the relevance of recombinant DNA techniques to this invention, one need not be confined to the sequences of amino acids found in natural antibodies; antibodies can be redesigned to obtain desired characteristics. The possible variations are many and range from the changing of just one or a few amino acids to the complete redesign of, for example, the constant region. Changes in the constant region will, in general, be made in order to improve the cellular process characteristics, such as complement fixation, interaction with membranes, and other effector functions. Changes in the variable region will be made in order to improve the antigen binding characteristics. The antibody can also be engineered so as to aid the specific delivery of a toxic agent according to the "magic bullet" concept. Alterations, can be made by standard recombinant techniques and also by oligonucleotide-directed mutagenesis techniques (Dalbadie-McFarland, et al *Proc. Natl. Acad. Sci.* (*USA*), 79:6,409 (1982)).

"Univalent antibodies" refers to aggregations which comprise a heavy chain light chain dimer bound to the Fc (or stem) region of a second heavy chain. Such antibodies are specific for antigen but have the additional desirable property of targeting tissues with specific antigenic surfaces, without causing its antigenic effectiveness to be impaired—i.e., there is no antigenic modulation. This phenomenon and the property of univalent antibodies in this regard is set forth in Glennie, M. J., et al., *Nature*, 295: 712 (1982). Univalent antibodies have heretofore been formed by proteolysis.

"Fab" region refers to those portions of the chains which are roughly equivalent, or analogous, to the sequences which comprise the Y branch portions of the heavy chain and to the light chain in its entirety, and which collectively (in aggregates) have been shown to exhibit antibody activity. "Fab protein", which protein is one of the aspects of the invention, includes aggregates of one heavy and one light chain (commonly known as Fab'), as well as tetramers which correspond to the two branch segments of the antibody Y, (commonly known as $F(ab)_2$), whether any of the above are covalently or non-covalently aggregated, so long as the aggregation is capable of selectively reacting with a particular antigen or antigen family. Fab antibodies have, as have univalent ones, been formed heretofore by proteolysis, and share the property of not eliciting antigen modulation on target tissues. However, as they lack the "effector" Fc: portion they cannot effect, for example, lysis of the target cell by macrophages.

"Fab protein" has similar subsets according to the definition of the present invention as does the general term "antibodies" or "immunoglobulins". Thus, "mammalian" Fab protein, "hybrid" Fab protein "chimeric" Fab and "altered" Fab protein are defined analogously to the corresponding definitions set forth in the previous paragraphs for the various types of antibodies.

Individual heavy or light chains may of course be "mammalian", "chimeric" or "altered" in accordance with the above. As will become apparent from the detailed description of the invention, it is possible, using the techniques disclosed to prepare other combinations of the four-peptide chain aggregates, besides those specifically defined, such as hybrid antibodies containing chimeric light and mammalian heavy chains, hybrid Fab proteins containing chimeric Fab proteins of heavy chains associated with mammalian light chains, and so forth.

"Expression vector" includes vectors which are capable of expressing DNA sequences contained therein, i.e., the coding sequences are operably linked to other sequences capable of effecting their expression. It is implied, although not always explicitly stated, that these expression vectors must be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. Clearly a lack of replicability would render them effectively inoperable. A useful, but not a necessary, element of an effective expression vector is a marker encoding sequence—i.e. a sequence encoding a protein which results in a phenotypic property (e.g. tetracycline resistance) of the cells containing the protein which permits those cells to be readily identified. In sum, "expression vector" is given a functional definition, and any DNA sequence which is capable of effecting expression of a specified contained DNA code is included in this term, as it is applied to the specified sequence. As at present, such vectors are frequently in the form of plasmids, thus "plasmid" and "expression vector" are often used interchangeably. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which may, from time to time become known in the art.

"Recombinant host cells" refers to cells which have been transformed with vectors constructed using recombinant DNA techniques. As defined herein, the antibody or modification thereof produced by a recombinant host cell is by virtue of this transformation, rather than in such lesser amounts, or more commonly, in such less than detectable amounts, as would be produced by the untransformed host.

In descriptions of processes for isolation of antibodies from recombinant hosts, the terms "cell" and "cell culture" are used interchangeably to denote the source of antibody unless it is clearly specified otherwise. In other words, recovery of antibody from the "cells" may mean either from spun down whole cells, or from the cell culture containing both the medium and the suspended cells.

B. Host Cell Cultures and Vectors

The vectors and methods disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms.

In general, of course, prokaryotes are preferred for cloning of DNA sequences in constructing the vectors useful in the invention. For example, *E. coli* K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include *E. coli* strains such as *E. coli* B, and *E. coli* X1776 (ATTC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes may also be used for expression. The aforementioned strains, as well as *E. coli* W3110 (F^-, λ^-, prototrophic, ATTC No. 27325), bacilli such as *Bacillus subtilus*, and other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various Pseudomonas species may be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (Bolivar, et al., *Gene* 2: 95 (1977)). PBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial

plasmid must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins. Those promoters most commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems Chang et al, *Nature* 275: 615 (1978); Itakura, et al, *Science* 198: 1056 (1977); (Goeddel et al, *Nature* 281: 544 (1979)) and a tryptophan (trp) promoter system (Goeddel, et al, *Nucleic kids Res.*, 8: 4057 (1980); EPO Appl Publ No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized., and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebenlist, et al, Cell 20: 269 (1980)).

In addition to prokaryates, eukaryotic microbes, such as yeast cultures may also be used. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example, (Stinchcomb et al, *Nature*, 282: 39 (1979); Kingsman et al, *Gene*, 7: 141 (1979); Tschemper, et al, *Gene*, 10: 157 (1980)) is commonly used. This plasmid already contains the trp1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, *Genetics*, 85: 12 (1977)). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman, et al., *J. Biol. Chem.*, 255: 2073 (1980)) or other glycolytic enzymes (Hess, et al, *J. Adv. Enzyme Reg.*, 7: 149 (1968); Holland, et al, *Biochemistry*, 17: 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization (Holland, ibid.). Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers, et al, *Nature*, 273: 113 (1978)) incorporated herein by reference. Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g. Polyoma, Adeno, VSV, BPV, etc.) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

It will be understood that this invention, although described herein in terms of a preferred embodiment, should not be construed as limited to those host cells, vectors and expression systems exemplified.

C. Methods Employed

C.1 Transformation

If cells without formidable cell wall barriers are used as host cells, transfection is carried out by the calcium phosphate precipitation method as described by Graham and Van der Eb, *Virology*, 52: 546 (1978). However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used.

If prokaryotic cells or cells which contain substantial cell wall constructions are used, the preferred method of transfection is calcium treatment using calcium chloride as described by Cohen, F. N. et al *Proc. Natl. Acad. Sci. (USA)* 69: 2110 (1972).

C.2 Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required. The methods employed are not dependent on the DNA source, or intended host.

Cleavage is performed by treating with restriction enzyme (or enzymes) in suitable buffer. In general, about 1 μg plasmid or DNA fragments is used with about 1 unit of enzyme in about 20 μl of buffer solution. (Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer.) Incubation times of about 1 hour at 37° C. are workable. After incubations, protein is removed by extraction with phenol and chloroform, and the nucleic acid is recovered from the aqueous fraction by precipitation with ethanol.

If blunt ends are required, the preparation is treated for 15 minutes at 15° with 10 units of *E. coli* DNA Polymerase I (Klenow), phenol-chloroform extracted, and ethanol precipitated.

Size separation of the cleaved fragments is performed using 6 percent polyacrylamide gel described by Goeddel, D., et al, *Nucleic Acids Res.*, 8: 4057 (1980) incorporated herein by reference.

For ligation, approximately equimolar amounts of the desired components, suitably end tailored to provide correct matching are treated with about 10 units T4 DNA ligase per 0.5 μg DNA. (When cleaved vectors are used as components, it may be useful to prevent religation of the cleaved vector by pretreatment with bacterial alkaline phosphatase.)

In the examples described below correct ligations for plasmid construction are confirmed by transforming *E. coli* K12 strain 294 (ATCC 31446) with the ligation mixture. Successful transformants were selected by ampicillin or tetracycline resistance depending on the mode of plasmid construction. Plasmids from the transformants were then prepared, analyzed by restriction and/or sequenced by the method of Messing, et al, *Nucleic Acids Res.*, 9:309 (1981) or by the method of Maxam, et al, *Methods in Enzymology*, 65:499 (1980).

D. Outline of Procedures

D.1 Mammalian Antibodies

The first type of antibody which forms a part of this invention, and is prepared by the methods thereof, is "mammalian antibody"-one wherein the heavy and light chains mimic the amino acid sequences of an antibody otherwise produced by a mature mammalian B lymphocyte either in situ or when fused with an immortalized cell as part of a hybridoma culture. In outline, these antibodies are produced as follows:

Messenger RNA coding for heavy or light chain is isolated from a suitable source, either mature B cells or a hybridoma culture, employing standard techniques of RNA isolation, and the use of oligo-dT cellulose chromatography to segregate the poly-A mRNA. The poly-A mRNA may, further, be fractionated to obtain sequences of sufficient size to code for the amino acid sequences in the light or heavy chain of the desired antibody as the case may be.

A cDNA library is then prepared from the mixture of mRNA using a suitable primer, preferably a nucleic acid sequence which is characteristic of the desired cDNA. Such a primer may be hypothesized and synthesized based on the amino acid sequence of the antibody if the sequence is known. In the alternative cDNA from unfractionated poly-A mRNA from a cell line producing the desired antibody or poly-dT may also be used. The resulting cDNA is optionally size fractionated on polyacrylamide gel and then extended with, for example, dC residues for annealing with pBR322 or other suitable cloning vector which has been cleaved by a suitable restriction enzyme, such as Pst I, and extended with dG residues. Alternative means of forming cloning vectors containing the cDNA using other tails and other cloning vector remainder may, of course, also be used but the foregoing is a standard and preferable choice. A suitable host cell strain, typically *E. coli*, is transformed with the annealed cloning vectors, and the successful transformants identified by means of, for example, tetracycline resistance or other phenotypic characteristic residing on the cloning vector plasmid.

Successful transformants are picked and transferred to microtiter dishes or other support for further growth and preservation. Nitrocellulose filter imprints of these growing cultures are then probed with suitable nucleotide sequences containing bases known to be complementary to desired sequences in the cDNA. Several types of probe may be used, preferably synthetic single stranded DNA sequences labeled by kinasing with ATP^{32}. The cells fixed to the nitrocellulose filter are lysed, the DNA denatured, and then fixed before reaction with kinased probe. Clones which successfully hybridize are detected by contact with a photoplate, then plasmids from the growing colonies isolated and sequenced by means known in the art to verify that the desired portions of the gene are present.

The desired gene fragments are excised and tailored to assure appropriate reading frame with the control segments when inserted into suitable expression vectors. Typically, nucleotides are added to the 5' end to include a start signal and a suitably positioned restriction endonuclease site.

The tailored gene sequence is then positioned in a vector which contains a promoter in reading frame with the gene and compatible with the proposed host cell. A number of plasmids such as those described in U.S. patent application Ser. Nos. 307,473; 291,892; and 305,657 (EPO Publ. Nos. 0036776; 0048970 and 0051873) have been described which already contain the appropriate promoters, control sequences, ribosome binding sites, and transcription termination sites, as well as convenient markers.

In the present invention, the gene coding for the light chain and that coding for the heaving chain are recovered separately by the procedures outlined above. Thus then may be inserted into separate expression plasmids, or together in the same plasmid, so long as each is under suitable promoter and translation control.

The expression vectors constructed above are then used to transform suitable cells. The light and heavy chains may be transformed into separate cell cultures, either of the same or of differing species; separate plasmids for light and heavy chain may be used to co-transform a single cell culture, or, finally, a single expression plasmid containing both genes and capable of expressing the genes for both light and heavy chain may be transformed into a single cell culture.

Regardless of which of the three foregoing options is chosen, the cells are grown under conditions appropriate to the production of the desired protein. Such conditions are primarily mandated by the type of promoter and control systems used in the expression vector, rather than by the nature of the desired protein. The protein thus produced is then recovered from the cell culture by methods known in the art, but choice of which is necessarily dependent on the form in which the protein is expressed. For example, it is common for mature heterologous proteins expressed in *E. coli* to be deposited within the cells as insoluble particles which require cell lysis and solubilization in denaturant to permit recovery. On the other hand, proteins under proper synthesis circumstances, in yeast and bacterial strains, can be secreted into the medium (yeast and gram positive bacteria) or into the periplasmic space (gram negative bacteria) allowing recovery by less drastic procedures. Tissue culture cells as hosts also appear, in general, to permit reasonably facile recovery of heterologous proteins.

When heavy and light chain are coexpressed in the same host, the isolation procedure is designed so as to recover reconstituted antibody. This can be accomplished in vitro as described below, or might be possible in vivo in a microorganism which secretes the IgG chains out of the reducing environment of the cytoplasm. A more detailed description is given in D.2, below.

D.2 Chain Recombination Techniques

The ability of the method of the invention to produce heavy and light chains or portions thereof, in isolation from each other offers the opportunity to obtain unique and unprecedented assemblies of immunoglobulins, Fab regions, and univalent antibodies. Such preparations require the use of techniques to reassemble isolated chains. Such means are known in the art, and it is, thus, appropriate to review them here.

While single chain disulfide bond containing proteins have been reduced and reoxidized to regenerate in high yield

native structure and activity (Freedman, R. B., et al. In *Enzymology of Post Translational Modification of Proteins,* I: 157–212 (1980) Academic Press, NY.), proteins which consist of discontinuous polypeptide chains held together by disulfide bonds are more difficult to reconstruct in vitro after reductive cleavage. Insulin, a cameo case, has received much experimental attention over the years, and can now be reconstructed so efficiently that an industrial process has been built around it (Chance, R. E., et al., In *Peptides: Proceedings of the Seventh Annual American Peptide Symposium* (Rich, D. H. and Gross. E., eds.) 721–728, Pierce Chemical Co., Rockford, Ill. (1981)).

Immunoglobulin has proved a more difficult problem than insulin. The tetramer is stabilized intra and intermolecularly by 15 or more disulfide bonds. It has been possible to recombine heavy and light chains, disrupted by cleavage of only the interchain disulfides, to regain antibody activity even without restoration of the inter-chain disulfides (Edelman, G. M., et al., *Proc. Natl. Acad. Sci. (USA)* 50: 753 (1963)). In addition, active fragments of IgG formed by proteolysis (Fab fragments of ~50,000 MW) can be split into their fully reduced heavy chain and light chain components and fairly efficiently reconstructed to give active antibody (Haber, E., *Proc. Natl. Acad. Sci. (USA)* 52: 1099 (1964); Whitney, P. L., et al., *Proc. Natl. Acad. Sci. (USA)* 53: 524 (1965)). Attempts to reconstitute active antibody from fully reduced native IgG have been largely unsuccessful, presumably due to insolubility of the reduced chains and of side products or intermediates in the refolding pathway (see discussion in Freedman, M. H., et al., *J. Biol. Chem.* 241: 5225 (1966)). If, however, the immunoglobulin is randomly modified by polyalanylation of its lysines before complete reduction, the separated chains have the ability to recover antigen-combining activity upon reoxidation (ibid).

A particularly suitable method for immunoglobulin reconstitution is derivable from the now classical insulin recombination studies, wherein starting material was prepared by oxidative sulfitolysis, thus generating thiol-labile S-sulfonate groups at all cysteines in the protein, non-reductively breaking disulfides (Chance et al. (supra)). Oxidative sulfitolysis is a mild disulfide cleavage reaction (Means, G. E., et al., *Chemical Modification of Proteins,* Holden-Day, San Francisco (1971)) which is sometimes more gentle than reduction, and which generates derivatives which are stable until exposed to mild reducing agent at which time disulfide reformation can occur via thiol-disulfide interchange. In the present invention the heavy and light chain S-sulfonates generated by oxidative sulfitolysis were reconstituted utilizing both air oxidation and thiol-disulfide interchange to drive disulfide bond formation. The general procedure is set forth in detail in U.S. Ser. No. 452,187, filed Dec. 22, 1982 (EPO Appln. No. 83.307840.5), incorporated herein by reference.

D.3 Variants Permitted by Recombinant Technology

Using the techniques described in paragraphs D.1 and D.2, additional operations which were utilized to gain efficient production of mammalian antibody can be varied in quite straightforward and simple ways to produce a great variety of modifications of this basic antibody form. These variations are inherent in the use of recombinant technology, which permits modification at a genetic level of amino acid sequences in normally encountered mammalian immunoglobulin chains, and the great power of this approach lies in its ability to achieve these variations, as well as in its potential for economic and specific production of desired scarce, and often contaminated, molecules. The variations also inhere in the ability to isolate production of individual chains, and thus create novel assemblies.

Briefly, since genetic manipulations permit reconstruction of genomic material in the process of construction of expression vectors, such reconstruction can be manipulated to produce new coding sequences for the components of "natural" antibodies or immunoglobulins. As discussed in further detail below, the coding sequence for a mammalian heavy chain may not be derived entirely from a single source or single species, but portions of a sequence can be recovered by the techniques described in D.1 from differing pools of mRNA, such as murine-murine hybridomas, human-murine hybridomas, or B cells differentiated in response to a series of antigen challenges. The desired portions of the sequences in each case can be recovered using the probe and analysis techniques described in D.1, and recombined in an expression vector using the same ligation procedures as would be employed for portions of the same model sequence. Such chimeric chains can be constructed of any desired length; hence, for example, a complete heavy chain can be constructed, or only sequence for the Fab region thereof.

The additional area of flexibility which arises from the use of recombinant techniques results from the power to produce heavy and light chains or fragments thereof in separate cultures or of unique combinations of heavy and light chain in the same culture, and to prevent reconstitution of the antibody or immunoglobulin aggregation until the suitable components are assembled. Thus, while normal antibody production results automatically in the formation of "mammalian antibodies" because the light and heavy chain portions are constructed in response to a particular determinant in the same cell, the methods of the present invention present the opportunity to assemble entirely new mixtures. Somewhat limited quantities of "hybrid" antibodies have been produced by "quadromas" i.e., fusions of two hybridoma cell cultures which permit random assemblies of the heavy and light chains so produced.

The present invention permits a more controlled assembly of desired chains, either by mixing the desired chains in vitro, or by transforming the same culture with the coding sequences for the desired chains.

D.4 Composite Immunoglobulins

The foregoing procedure, which describes in detail the recombinant production of mammalian antibodies is employed with some modifications to construct the remaining types of antibodies or NSIs encompassed by the present invention. To prepare the particular embodiment of composite non-specific immunoglobulin wherein the homology of the chains corresponds to the sequences of immunoglobulins of different specificities, it is of course, only necessary to prepare the heavy and light chains in separate cultures and reassemble them as desired.

For example, in order to make an anti-CEA light chain/antihepatitis heavy chain composite antibody, a suitable source for the mRNA used as a template for the light chain clone would comprise, for instance, the anti CEA producing cell line of paragraph E.1. The mRNA corresponding to heavy chain would be derived from B cells raised in response to hepatitis infection or from hybridoma in which the B cell was of this origin. It is clear that such composites can be assembled using the methods of the invention almost at will, and are limited only by available sources of mRNA suitable for use as templates for the respective chains. All other features of the process are similar to those described above.

D.5 Hybrid Antibodies

Hybrid antibodies are particularly useful as they are capable of simultaneous reaction with more than one antigen. Pairs of heavy and light chains corresponding to chains

of antibodies for different antigens, such as those set forth in paragraph D.4 are prepared in four separate cultures, thus preventing premature assembly of the tetramer. Subsequent mixing of the four separately prepared peptides then permits assembly into the desired tetramers. While random aggregation may lead to the formation of considerable undesired product, that portion of the product in which homologous light and heavy chains are bound to each other and mismatched to another pair gives the desired hybrid antibody.

D.6 Chimeric Antibodies

For construction of chimeric antibodies (wherein, for example, the variable sequences are separately derived from the constant sequences) the procedures of paragraph C.1 and D.2 are again applicable with appropriate additions and modifications. A preferred procedure is to recover desired portions of the genes encoding for parts of the heavy and light chains from suitable, differing, sources and then to religate these fragments using restriction endonucleases to reconstruct the gene coding for each chain.

For example, in a particularly preferred chimeric construction, portions of the heavy chain gene and of the light chain gene which encode the variable sequences of antibodies produced by a murine hybridoma culture are recovered and closed from this culture and gene fragments encoding the constant regions of the heavy and light chains for human antibodies recovered and cloned from, for example, human myeloma cells. Suitable restriction enzymes may then be used to ligate the variable portions of the mouse gene to the constant regions of the human gene for each of the two chains. The chimeric chains are produced as set forth in D.1, aggregated as set forth in D.2 and used in the same manner as the non-chimeric forms. Of course, any splice point in the chains can be chosen.

D.7 Altered Antibodies

Altered antibodies present, in essence, an extension of chimeric ones. Again, the techniques of D.1 and D.2 are applicable; however, rather than splicing portions of the chain(s), suitable amino acid alterations, deletions or additions are made using available techniques such as mutagenesis (supra). For example, genes which encode antibodies having diminished complement fixation properties, or which have enhanced metal binding capacities are prepared using such techniques. The latter type may, for example, take advantage of the known gene sequence encoding metallothionein II (Karin, M., et al., *Nature*, 299: 797 (1982)). The chelating properties of this molecular fragment are useful in carrying heavy metals to tumor sites as an aid in tumor imaging (Scheinberg, D. A., et al., *Science*, 215: 19 (1982).

D.8 Univalent Antibodies

In another preferred embodiment, antibodies are formed which comprise one heavy and light chain pair coupled with the Fc region of a third (heavy) chain. These antibodies have a particularly useful property. They can, like ordinary antibodies, be used to target antigenic surfaces of tissues, such as tumors, but, unlike ordinary antibodies, they do not cause the antigenic surfaces of the target tissue to retreat and become non-receptive. Ordinary antibody use results in aggregation and subsequent inactivation, for several hours, of such surface antigens.

The method of construction of univalent antibodies is a straightforward application of the invention. The gene for heavy chain of the desired Fc region is cleaved by restriction enzymes, and only that portion coding for the desired Fc region expressed. This portion is then bound using the technique of D.2 to separately produced heavy chain the desired pairs separated from heavy heavy and Fc Fc combinations, and separately produced light chain added. Pre-binding of the two heavy chain portions thus diminishes the probability of formation of ordinary antibody.

D.9 Fab Protein

Similarly, it is not necessary to include the entire gene for the heavy chain portion. All of the aforementioned variations can be superimposed on a procedure for Fab protein production and the overall procedure differs only in that the portion of the heavy chain coding for the amino terminal **220** amino acids is employed in the appropriate expression vector.

E. Specific Examples of Preferred Embodiments

The invention has been described above in general terms and there follow several specific examples of embodiments which set forth details of experimental procedure in producing the desired antibodies. Example E.1 sets forth the general procedure for preparing anti CEA antibody components, i.e. for a "mammalian antibody". Example E.3 sets forth the procedure for reconstitution and thus is applicable to preparation of mammalian, composite, hybrid and chimeric immunoglobulins, and Fab proteins and univalent antibodies. Example E.4 sets forth the procedure for tailoring the heavy or light chain so that the variable and constant regions may be derived from different sources. Example E.5 sets forth the method of obtaining a shortened heavy chain genome which permits the production of the Fab regions and, in an analogous manner, Fc region.

The examples set forth below are included for illustrative purposes and do not limit the scope of the invention.

E.1 Construction of Expression Vectors for Murine anti-CEA Antibody Chains and Peptide Synthesis

Carcinoembryonic antigen (CEA) is associated with the surface of certain tumor cells of human origin (Gold, P., et al., *J. Exp. Med.*, 122: 467 (1965)). Antibodies which bind to CEA (anti-CEA antibodies) are useful in early detection of these tumors (Van Nagell, T. R., et al., *Cancer Res.* 40: 502 (1980)), and have the potential for use in treatment of those human tumors which appear to support CEA at their surfaces. A mouse hybridoma cell line which secretes anti-CEA antibodies of the IgG_1 class, CEA.66-E3, has been prepared as described by Wagener, C, et al., *J. Immunol.* (in press) which is incorporated herein by reference, and was used as mRNA source. The production of anti CEA antibodies by this cell line was determined. The N-terminal sequences of the antibodies produced by these cells was compared with those of monoclonal anti CEA as follows. Purified IgG was treated with PCAse (Podell, D. N., et al., BBRC 81: 176 (1978)), and then dissociated in 6M guanidine hydrochloride, 10 mM 2-mercaptoethanol (1.0 mg of immunoglobulin, 5 min, 100° C. water bath). The dissociated chains were separated on a Waters Associates alkyl phenyl column using a linear gradient from 100 percent A (0.1 percent TFA-water) to 90 percent B (TFA/H_2O/MeCN 0.1/9.9/90) at a flow rate of 0.8 ml/min. Three major peaks were eluted and analyzed on SDS gels by silver staining. The first two peaks were pure light chain (MW 25,000 daltons), the third peak showed a (7:3) mixture of heavy and light chain. 1.2 nmoles of light chain were sequenced by the method of Shively, J. E., *Methods in Enzymology*, 79: 31 (1981), with an NH_2-terminal yield of 0.4 nmoles. A mixture of heavy and light chains (3 nmoles) was also sequenced, and sequence of light chain was deducted from the double sequence to yield the sequence of the heavy chain.

In the description which follows, isolation and expression of the genes for the heavy and light chains for anti CEA antibody produced by CEA.66-E3 are described. As the constant regions of these chains belong to the gamma and kappa families, respectively, "light chain" and "kappa

chain", and "heavy chain" and "gamma chain", respectively, are used interchangeably below.

E.1.1 Isolation of Messenger RNA for Anti CEA Light and Heavy (Kappa and Gamma) Chains

Total RNA from CEA.66-E3 cells was extracted essentially as reported by Lynch et al, *Virology*, 98: 251 (1979). Cells were pelleted by centrifugation and approximately 1 g portions of pellet resuspended in 10 ml of 10 mM NaCl, 10 mM Tris HCl (pH 7.4), 1.5 mM $MgCl_2$. The resuspended cells were lysed by addition of non-ionic detergent NP-40 to a final concentration of 1 percent, and nuclei removed by centrifugation. After addition of SDS (pH 7.4) to 1 percent final concentration, the supernatant was extracted twice with 3 ml portions of phenol (redistilled)/chloroform: isoamyl alcohol 25:1 at 4° C. The aqueous phase was made 0.2 M in NaCl and total RNA was precipitated by addition of two volumes of 100 percent ethanol and overnight storage at −20° C. After centrifugation, polyA mRNA was purified from total RNA by oligo-dT cellulose chromatography as described by Aviv and Leder, *Proc. Nat'l, Acad. Sci. (USA)*, 69: 1408 (19672), 142 μg of polyA mRNA was obtained from 1 g cells.

E.1.2 Preparation of *E. coli* Colony Library Containing Plasmids with Heavy and Light DNA Sequence Inserts

5 μg of the unfractionated polyA mRNA prepared in paragraph E.1.1 was used as template for oligo-dT primed preparation of double-stranded (ds) cDNA by standard procedures as described by Goeddel et al., *Nature* 281: 544 (1979) and Wickens et al., *J. Biol. Chem.* 253: 2483 (1978) incorporated herein by reference. The cDNA was size fractionated by 6 percent polyacrylamide gel electrophoresis and 124 ng of ds cDNA greater than 600 base pairs in length was recovered by electroelution. A 20 ng portion of ds cDNA was extended with deoxy C residues using terminal deoxynucleotidyl transferase as described in Chang et al., *Nature* 275: 617 (1978) incorporated herein by reference, and annealed with 200 ng of the plasmid pBR322 (Bolivar et al., *Gene* 2: 95 (1977)) which had been cleaved with Pst I and tailed with deoxy G. Each annealed mixture was then transformed into *E. coli* K12 strain 294 (ATCC No. 31446). Approximately 8500 ampicillin sensitive, tetracycline resistant transformants were obtained.

E.1.3 Preparation of Synthetic Probes

The 14mer, 5' GGTGGGAAGATGGA 3' complementary to the coding sequence of constant region for mouse MOPC21 kappa chain which begins 25 basepairs 3' of the variable region DNA sequence was used as kappa chain probe. A 15 mer, 5' GACCAGGCATCCCAG 3', complementary to a coding sequence located 72 basepairs 3' of the variable region DNA sequence for mouse MOPC21 gamma chain was used to probe gamma chain gene.

Both probes were synthesized by the phosphotriester method described in German Offenlegungschrift 2644432, incorporated herein by reference, and made radioactive by kinasing as follows: 250 ng of deoxyoligonucleotide were combined in 25 μl of 60 mM Tris HCl (pH 8), 10 mM $MgCl_2$, 15 mM beta-mercaptoethanol, and 100 μCi (γ-^{32}P) ATP (Amersham, 5000 Ci/mMole). 5 units of T4 polynucleotide kinase were added and the reduction was allowed to proceed at 37° C. for 30 minutes and terminated by addition of EDTA to 20 mM.

E.1.4 Screening of Colony Library for Kappa or Gamma Chain Sequences

~2000 colonies prepared as described in paragraph E.1.2 were individually inoculated into wells of microtitre dishes containing LB (Miller, Experiments in Molecular Genetics, p. 431–3, Cold Spring Harbor, Lab., Cold Spring Harbor, N.Y. (1972))+5 μg/ml tetracycline and stored at −20° C. after addition of DMSO to 7 percent. Individual colonies from this library were transferred to duplicate sets of Schleicher and Schuell BA85/20 nitrocellulose filters and grown on agar plates containing LB+5 μg/ml tetracycline. After ~10 hours growth at 37° C. the colony filters were transferred to agar plates containing LB+5 μg/ml tetracycline and 12.5 μg/ml chloramphenicol and reincubated overnight at 37° C. The DNA from each colony was then denatured and fixed to the filter by a modification of the Grunstein-Hogness procedure as described in Grunstein et al., *Proc. Natl. Acad. Sci. (USA)* 72: 3961 (1975), incorporated herein by reference. Each filter was floated for 3 minutes on 0.5 N NaOH, 1.5 M NaCl to lyse the colonies and denature the DNA then neutralized by floating for 15 minutes on 3 M NaCl, 0.5 M Tris HCl (pH 7.5). The filters were then floated for an additional 15 minutes on 2XSSC, and subsequently baked for 2 hours in an 80° C. vacuum oven. The filters were prehybridized for ~2 hours at room temperature in 0.9 M NaCl, 1X Denhardts, 100 mM Tris HCl (pH 7.5), 5 mM Na-EDTA, 1 mM ATP, 1 M sodium phosphate (dibasic), 1 mM sodium pyrophosphate, 0.5 percent NP-40, and 200 μg/ml *E. coli* t-RNA, and hybridized in the same solution overnight, essentially as described by Wallace et al. *Nucleic Acids Research* 9: 879 (1981) using ~40×10^6 cpm of either the kinased kappa or gamma probe described above.

After extensive washing at 37° C. in 6X SSC, 0.1 percent SDS, the filters were exposed to Kodak XR-5 X-ray film with DuPont Lightning-Plus intensifying screens for 16–24 hours at −80° C. Approximately 20 colonies which hybridized with kappa chain probe and 20 which hybridized with gamma chain probe were characterized.

E.1.5 Characterization of Colonies which Hybridize to Kappa DNA Sequence Probe

Plasmid DNAs isolated from several different transformants which hybridized to kappa chain probe were cleaved with Pst I and fractionated by polyacrylamide gel electrophoresis (PAGE). This analysis demonstrated that a number of plasmid DNAs contained cDNA inserts large enough to encode full length kappa chain. The complete nucleotide sequence of the cDNA insert of one of these plasmids was determined by the dideoxynucleotide chain termination method as described by Smith, *Methods Enzymol.* 65, 560 (1980) incorporated herein by reference after subcloning restriction endonuclease cleavage fragments into M13 vectors (Messing et al., *Nucleic Acids Research* 9: 309 (1981). FIG. 2 shows the nucleotide sequence of the cDNA insert of pK17G4 and FIG. 3 shows the gene sequence with the corresponding amino acid sequence. Thus, the entire coding region of mouse anti-CEA kappa chain was isolated on this one large DNA fragment. The amino acid sequence of kappa chain, deduced from the nucleotide sequence of the pK17G4 cDNA insert, corresponds perfectly with the first 23 N-terminal amino acids of mature mouse anti-CEA kappa chain as determined by amino acid sequence analysis of purified mouse anti-CEA kappa chain. The coding region of pK17G4 contains 27 basepairs or 9 amino acids of the presequence and 642 basepairs or 214 amino acids of the mature protein. The mature unglycosylated protein (MW 24,553) has a variable region of 119 amino acids, including the J1 joining region of 12 amino acids, and a constant region of 107 amino acids. After the stop codon behind amino acids 215 begins 212 basepairs of 3' untranslated sequence up to the polyA addition. The kappa chain probe used to identify pK17G4 hybridizes to nucleotides 374–388 (FIG. 2).

E.1.6 Characterization of Colonies which Hybridize to Gamma 1 DNA Probe

Plasmid DNA isolated from several transformants positive for hybridization with the heavy chain gamma 1 probe was subjected to Pst I restriction endonuclease analysis as described in E.1.5. Plasmid DNAs demonstrating the largest cDNA insert fragments were selected for further study. Nucleotide sequence coding for mouse heavy (gamma-1) chain, shows an NcoI restriction endonuclease cleavage site near the junction between variable and constant region. Selected plasmid DNAs were digested with both PstI and NcoI and sized on polyacrylamide. This analysis allowed identification of a number of plasmid DNAs that contain NcoI restriction endonuclease sites, although none that demonstrate cDNA insert fragments large enough to encode the entire coding region of mouse anti-CEA heavy chain.

In one plasmid isolated, p γ298 the cDNA insert of about 1300 bp contains sequence information for the 5' untranslated region, the signal sequence and the N-terminal portion of heavy chain. Because pγ298 did not encode the C-terminal sequence for mouse anti-CEA gamma 1 chain, plasmid DNA was isolated from other colonies and screened with PstI and NcoI. The C-terminal region of the cDNA insert of pγ11 was sequence and shown to contain the stop codon, 3' untranslated sequence and that portion of the coding sequence missing from p γ298.

FIG. **4** presents the entire nucleotide sequence of mouse anti-CEA heavy chain (as determined by the dideoxynucleotide chain termination method of Smith, *Methods Enzymol.*, 65: 560 (1980)) and FIG. **5** includes the translated sequence.

The amino acid sequence of gamma 1 (heavy chain) deduced from the nucleotide sequence of the pγ298 cDNA insert corresponds perfectly to the first 23 N-terminal amino acids of mature mouse anti-CEA gamma 1 chain as determined by amino acid sequence analysis of purified mouse anti-CEA gamma-1 chain. The coding region consists of 57 basepairs or 19 amino acids of presequences and 1346 basepairs or 447 amino acids of mature protein. The mature unglycosolated protein (MW 52,258) has a variable region of 135 amino acids, including a D region of 12 amino acids, and a J4 joining region of 13 amino acids. The constant region is 324 amino acids. After the stop codon behind amino acid 447 begins 96 bp of 3' untranslated sequences up to the polyA addition. The probe used to identify pγ298 and pγ11 hybridized to nucleotides 528–542 (FIG. **4**).

E.1.7 Construction of a Plasmid for Direct Expression of Mouse Mature Anti-CEA Kappa Chain Gene, pKCEAtrp207-1*

FIG. **6** illustrated the construction of pKCEAtrp207-1*

First, an intermediate plasmid pHGH207-1*, having a single trp promoter, was prepared as follows:

The plasmid pHGH 207 (described in U.S. patent application Ser. No. 307,473, filed Oct. 1, 1981 (EPO Publn. No. (0036776)) has a double lac promoter followed by the trp promoter, flanked by EcoR I sites and was used to prepare pHGH207-1. pHGH207 was digested with BamH 1, followed by partial digestion with EcoR I. The largest fragment, which contains the entire trp promoter, was isolated and ligated to the largest EcoR I- BamH I fragment from pBR322, and the ligation mixture used to transform *E. coli* 294. Tet^R Amp^R colonies were isolated, and most of them contained pHGH207-1. pHGH207-1* which lacks the EcoR1 site between the amp^R gene and the trp promoter, was obtained by partial digestion of pHGH207-1 with EcoR I, filling in the ends with Klenow and dNTPs, and religation.

5 μg of pHGH207-1* was digested with EcoRI, and the ends extended to blunt ends using 12 units of DNA Polymerase I in a 50 μl reaction containing 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4) and 1 mM in each dNTP at 37° C. for 1 hour, followed by extraction with phenol/$CHCl_3$ and precipitation with ethanol. The precipitated DNA was digested with BamH I, and the large vector fragment (fragment 1) purified using 5 percent polyacrylamide gel electrophoresis, electroelution, phenol/$CHCl_3$ extraction and ethanol precipitation.

The DNA was resuspended in 50 μl of 10 mM Tris pH 8, 1 mM EDTA and treated with 500 units Bacterial Alkaline Phosphatase (BAP) for 30' at 65° followed by phenol/$CHCl_3$ extraction and ethanol precipitation.

A DNA fragment containing part of the light chain sequence was prepared as follows: 7 μg of pH17G4 DNA was digested with Pst I and the kappa chain containing cDNA insert was isolated by 6 percent gel electrophoresis, and electroelution. After phenol/$CHCl_3$ extraction, ethanol precipitation and resuspension in water, this fragment was digested with Ava II. The 333 bp Pst I-Ava II DNA fragment was isolated and purified from a 6 percent polyacrylamide gel.

A 15 nucleotide DNA primer was synthesized by the phosphotriester method G. O. 2,644,432 (supra) and has the following sequence:

```
Met Asp Ile Val Met
5' ATG GAC ATT GTT ATG 3'
```

The 5' methionine serves as the initiation codon. 500 ng of this primer was phosphorylated at the 5' end with 10 units T4 DNA kinase in 20 μl reaction containing 0.5 mM ATP. ~200 ng of the Pst I-Ava II DNA fragment was mixed with the 20 μl of the phosphorylated primer, heated to 95° C. for 3 minutes and quick frozen in a dry-ice ethanol bath. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C. this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, and digested to completion with Sau 3A. The reaction mixture was then electrophoresed on a 6 percent polyacrylamide gel and ~50 ng of the 182 basepair amino-terminal blunt-end to Sau 3A fragment (fragment 2) was obtained after electroelution.

100 ng of fragment 1 (supra) and 50 ng of fragment 2 were combined in 20 μl of 20 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 2.5 mM ATP and 1 unit of T4 DNA ligase. After overnight ligation at 14° C. the reaction was transformed into *E. coli* K12 strain 294. Restriction endonuclease digestion of plasmid DNA from a number of ampicillin resistant transformants indicated the proper construction and DNA sequence analysis proved the desired nucleotide sequence through the initiation codon of this new plasmid, pKCEAlnt1 (FIG. **6**).

The remainder of the coding sequence of the kappa light chain gene was prepared as follows:

The Pst I cDNA insert fragment from 7 μg of K17G4 DNA was partially digested with Ava II and the Ava II cohesive ends were extended to blunt ends in a DNA Polymerase I large fragment reaction. Following 6 percent polyacrylamide gel electrophoresis the 686 basepair Pst I to blunt ended Ava II DNA fragment was isolated, purified and subjected to Hpa II restriction endonuclease digestion. The 497 basepair Hpa II to blunt ended Ava II DNA fragment (fragment 3) was isolated and purified after gel electrophoresis.

10 μg of pKCEAlnt1 DNA was digested with Ava I, extended with DNA polymerase I large fragment, and

digested with Xba I. Both the large blunt ended Ava I to Xba I vector fragment and the small blunt ended Ava I to Xba I fragment were isolated and purified from a 6 percent polyacrylamide gel after electrophoresis. The large vector fragment (fragment 4) was treated with Bacterial Alkaline Phosphatase (BAP), and the small fragment was digested with Hpa II, electrophoresed on a 6 percent polyacrylamide and the 169 basepair Xba I-Hpa II DNA fragment (fragment 5) was purified. ~75 ng of fragment 4, ~50 ng of fragment 3 and ~50 ng of fragment 5 were combined in a T4 DNA ligase reaction and incubated overnight at 14°, and the reaction mixture transformed into *E. coli* K12 strain 294. Plasmid DNA from six ampicillin resistant transformants were analyzed by restriction endonuclease digestion. One plasmid DNA demonstrated the proper construction and was designated pKCEAInt2.

Final construction was effected by ligating the K-CEA fragment, including the trp promoter from pKCEAInt2 into pBR322(XAP). (pBR322(XAP) is prepared as described in U.S. application Ser. No. 452,227, filed Dec. 22, 1982; from pBR322 by deletion of the AvaI-PvuII fragment followed by ligation.)

The K-CEA fragment was prepared by treating pKCEAInt2 with Ava I, blunt ending with DNA polymerase I (Klenow fragment) in the presence of DNTPs, digestion with Pst I and isolation of the desired fragment by gel electrophoresis and electroelution.

The large vector fragment from pBR322(XAP) was prepared by successive treatment with EcoR I, blunt ending with polymerase, and redigestion with Pst I, followed by isolation of the large vector fragment by electrophoresis and electroelution.

The K-CEA and large vector fragments as prepared in the preceding paragraphs were ligated with T4 DNA ligase, and the ligation mixture transformed into *E. coli* as above. Plasmid DNA from several ampicillin resistant transformants were selected for analysis, and one plasmid DNA demonstrated the proper construction, and was designated pKCEAtrp207-1*.

E.1.8 Construction of a Plasmid Vector for Direct Expression of Mouse Mature Anti-CEA Heavy (Gamma 1) Chain Gene, pγCEAtrp207-1*

FIG. 7 illustrates the construction of pγCEAtrp207-1*. This plasmid was constructed in two parts beginning with construction of the C-terminal region of the gamma 1 gene.

5 μg of plasmid pHGH207-1* was digested with Ava I, extended to blunt ends with DNA polymerase I large fragment (Klenow fragment), extracted with phenol/$CHCl_3$, and ethanol precipitated. The DNA was digested with BamH I treated with BAP and the large fragment (fragment A) was purified by 6 percent polyacrylamide gel electrophoresis and electroelution.

~5 μg of pγ11 was digested with Pst I and the gamma chain cDNA insert fragment containing the C-terminal portion of the gene was purified, digested with Ava II followed by extension of the Ava II cohesive ends with Klenow, followed by Taq I digestion. The 375 basepair blunt ended Ava II to Taq I fragment (fragment B) was isolated and purified by gel electrophoresis and electroelution.

9 μg of pγ298 was digested with Taq I and BamH I for isolation of the 496 basepair fragment (fragment C).

Approximately equimolar amounts of fragments A, B, and C were ligated overnight at 14° in 20 μl reaction mixture, then transformed into *E. coli* strain 294. The plasmid DNA from six ampicillin resistant transformants was committed to restriction endonuclease analysis and one plasmid DNA, named pγCEAInt, demonstrated the correct construction of the C-terminal portion of gamma 1 (FIG. 5).

To obtain the N-terminal sequences, 30 μg of pγ298 was digested with Pst I and the 628 basepair DNA fragment encoding the N-terminal region of mouse anti-CEA gamma chain was isolated and purified. This fragment was further digested with Alu I and Rsa I for isolation of the 280 basepair fragment. A 15 nucleotide DNA primer

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    met glu val met leu
5' ATG GAA GTG ATG CTG 3'
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was synthesized by the phosphotriester method (supra).

The 5' methionine serves as the initiation codon. 500 ng of this synthetic oligomer primer was phosphorylated at the 5' end in a reaction with 10 units T4 DNA kinase containing 0.5 mM ATP in 20 μl reaction mixture. ~500 ng of the 280 basepair Alu I-Rsa I DNA fragment was mixed with the phosphorylated primer. The mixture was heat denatured for 3 minutes at 95° and quenched in dry-ice ethanol. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C., this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, and digested to completion with HpaII. ~50 ng of the expected 125 basepair blunt-end to Hpa II DNA fragment (fragment D) was purified from the gel.

A second aliquot of pγ298 DNA was digested with Pst I, the 628 basepair DNA fragment purified by polyacrylamide gel electrophoresis, and further digested with BamH I and Hpa II. The resulting 380 basepair fragment (fragment E) was purified by gel electrophoresis.

~5 μg of pγCEAIntI was digested with EcoR I, the cohesive ends were made flush with DNA polymerase I (Klenow), further digested with BamH I, treated with BAP and electrophoresed on a 6 percent polyacrylamide gel. The large vector fragment (fragment F) was isolated and purified.

In a three fragment ligation, 50 ng fragment D, 100 ng fragment E, and 100 ng fragment F were ligated overnight at 4° in a 20 μl reaction mixture and used to transform *E. coli* K12 strain 294. The plasmid DNAs from 12 ampicillin resistant transformants were analyzed for the correct construction and the nucleotide sequence surrounding the initiation codon was verified to be correct for the plasmid named pγCEAInt2.

The expression plasmid, pγCEAtrp207-1* used for expression of the heavy chain gene is prepared by a 3-way ligation using the large vector fragment from pBR322(XAP) (supra) and two fragments prepared from pγCEAInt2.

pBR322(XAP) was treated as above by digestion with EcoR1, blunt ending with DNA polymerase (Klenow) in the presence of dNTPs, followed by digestion with Pst I, and isolation of the large vector fragment by gel electrophoresis. A 1543 base pair fragment from pγCEAInt2 containing trp promoter linked with the N-terminal coding region of the heavy chain gene was isolated by treating pγCEAInt2 with Pst I followed by BamH I, and isolation of the desired fragment using PAGE. The 869 base pair fragment containing the C-terminal coding portion of the gene was prepared by partial digestion of pγCEAInt2 with Ava I, blunt ending with Klenow, and subsequent digestion with BamH I, followed by purification of the desired fragment by gel electrophoresis.

The aforementioned three fragments were then ligated under standard conditions using T4 DNA ligase, and a ligation mixture used to transform *E. coli* strain 294. Plasmid DNAs from several tetracycline resistant transformants were analyzed; one plasmid DNA demonstrated the proper construction and was designated pγCEAtrp207-1*.

E.1.9 Production of Immunoglobulin Chains by *E. coli*

E. coli strain W3110 (ATTC No. 27325) was transformed with pγCEAtrp207-1* or pKCEAtrp207-1* using standard techniques.

To obtain double transformants, *E. coli* strain W3110 cells were transformed with a modified pKCEAtrp207-1*, pKCEAtrp207-1*Δ, which had been modified by cleaving a Pst 1-Pvu I fragment from the amp^R gene and religating. Cells transformed with pKCEAtrp207-1*Δ are thus sensitive to ampicillin but still resistant to tetracycline. Successful transformants were retransformed using pγCEAInt2 which confers resistance to ampicillin but not tetracycline. Cells containing both pKCEAtrp207-1*Δ and pγCEAInt2 thus identified by growth in a medium containing both ampicillin and tetracycline.

To confirm the production of heavy and/or light chains in the transformed cells, the cell samples were inoculated into M9 tryptophan free medium containing 10 μg/ml tetracycline, and induced with indoleacrylic acid (IAA) when the OD 550 reads 0.5. The induced cells were grown at 37° C. during various time periods and then spun down, and suspended in TE buffer containing 2 percent SDS and 0.1 M β-mercaptoethanol and boiled for 5 minutes. A 10xvolume of acetone was added and the cells kept at 22° C. for 10 minutes, then centrifuged at 12,000 rpm. The precipitate was suspended in O'Farrell SDS sample buffer (O'Farrell, P. H., *J. Biol. Chem.*, 250: 4007 (1975)); boiled 3 minutes, recentrifuged, and fractionated using SDS PAGE (10 percent), and stained with silver stain (Goldman, D. et al., *Science* 211: 1437 (1981)); or subjected to Western blot using rabbit anti-mouse IgG (Burnett, W. N., et al., *Anal. Biochem.* 112: 195 (1981)), for identification light chain and heavy chain.

Cells transformed with pγCEAtrp207-1* showed bands upon SDS PAGE corresponding to heavy chain molecular weight as developed by silver stain. Cells transformed with pKCEAtrp207-1* showed the proper molecular weight band for light chain as identified by Western blot; double transformed cells showed bands for both heavy and light chain molecular weight proteins when developed using rabbit anti-mouse IgG by Western blot. These results are shown in FIGS. **8A**, **8B**, and **8C**.

FIG. **8A** shows results developed by silver stain from cells transformed with pγCEAtrp207-1*. Lane 1 is monoclonal anti-CEA heavy chain (standard) from CEA.66-E3. Lanes 2b–5b are timed samples 2 hrs, 4 hrs, 6 hrs, and 24 hrs after IAA addition. Lanes 2a–5a are corresponding untransformed controls; Lanes 2c–5c are corresponding uninduced transformants.

FIG. **8B** shows results developed by Western blot from cells transformed with pKCEAtrp207-1*. Lanes 1b–6b are extracts from induced cell immediately, 1 hr, 3.5 hrs, 5 hrs, 8 hrs, and 24 hrs after IAA addition, and 1a–6a corresponding uninduced controls. Lane 7 is an extract from a pγCEAtrp207-1* control, lanes 8, 9, and 10 are varying amounts of anti CEA-kappa chain from CEA.66-E3 cells.

FIG. **8C** shows results developed by Western blot from four colonies of double transformed cells 24 hours after IAA addition (lanes 4–7). Lanes 1–3 are varying amounts of monoclonal gamma chain controls, lanes 8 and 9 are untransformed and pγCEAtrp207-1* transformed cell extracts, respectively.

In another quantitative assay, frozen, transformed *E. coli* cells grown according to E.1.10 (below) were lysed by heating in sodium dodecyl sulfate (SDS) β-mercaptoethanol cell lysis buffer at 100°. Aliquots were loaded on an SDS polyacrylamide gel next to lanes loaded with various amounts of hybridoma anti-CEA. The gel was developed by the Western blot, Burnett (supra), using ^{125}I-labeled sheep anti-mouse IgG antibody from New England Nuclear. The results are shown in FIG. **9**. The figure shows that the *E. coli* products co-migrate with the authentic hybridoma chains, indicating no detectable proteolytic degradation in *E. coli*. Heavy chain from mammalian cells is expected to be slightly heavier than *E. coli* material due to glycosylation in the former. Using the hybridoma lanes as a standard, the following estimates of heavy and light chain production were made:

	(Per gram of cells)
E. coli (W3110/pγCEAtrp207-1*)	5 mg γ
E. coli (W3110/pKCEAtrp207-1*)	1.5 mg K
E. coli (W3110/pKCEAtrp207-1*Δ, pγCEAInt2)	0.5 mg K, 1.0 mg γ

E.1.10 Reconstitution of Antibody from Recombinant K and Gamma Chains

In order to obtain heavy and light chain preparations for reconstitution, transformed cells were grown in larger batches, harvested and frozen. Conditions of growth of the variously transformed cells were as follows:

E. coli (W3110/pγCEAtrp207-1*) were inoculated into 500 ml LB medium containing 5 μg/ml tetracycline and grown on a rotary shaker for 8 hours. The culture was then transferred to 10 liters of fermentation medium containing yeast nutrients, salts, glucose, and 2 μg/ml tetracycline. Additional glucose was added during growth and at OD 550=20, indoleacrylic (IAA), a trp derepressor, was added to a concentration of 50 μg/ml. The cells were fed additional glucose to a final OD 550=40, achieved approximately 6 hours from the IAA addition.

E. coli (W3110) cells transformed with pKCEA trp 207-1* and double transformed (with pKCEAtrp207-1*Δ and pγCEAInt2) were grown in a manner analogous to that described above except that the OD 550 six hours after IAA addition at harvest was 25–30.

The cells were then harvested by centrifugation, and frozen.

E.2 Assay Method for Reconstituted Antibody

Anti-CEA activity was determined by ELISA as a criterion for successful reconstitution. Wells of microtiter plates (Dynatech Immulon) were saturated with CEA by incubating 100 μl of 2–5 μg CEA/ml solution in 0.1M carbonate buffer, pH 9.3 for 12 hours at room temperature. The wells were then washed 4 times with phosphate buffered saline (PBS), and then saturated with BSA by incubating 200 μl of 0.5 percent BSA in PBS for 2 hours at 37° C., followed by washing 4 times with PBS. Fifty microliters of each sample was applied to each well. A standard curve (shown in FIG. **10**), was run, which consisted of 50 μl samples of 10 μg, 5 μg, 1 μg, 500 ng, 100 ng, 50 ng, 10 ng, 5 ng and 1 ng anti-CEA/ml in 0.5 percent BSA in PBS, plus 50 μl of 0.5 percent BSA in PBS alone as a blank. All of the samples were incubated in the plate for 90 minutes at 37° C.

The plates were then washed 4 times with PBS, and sheep anti-mouse IgG-alkaline phosphate (TAGO, Inc.) was applied to each well by adding 100 μl of an enzyme concentration of 24 units/ml in 0.5 percent BSA in PBS. The solution was incubated at 37° C. for 90 minutes. The plates were washed 4 times with PBS before adding the substrate, 100 μl of a 0.4 mg/ml solution of p-nitrophenylphosphate (Sigma) in ethanolamine buffered saline, pH 9.5. The substrate was incubated 90 minutes at 37° C. for color development.

The A_{450} of each well was read by the Microelisa Auto Reader (Dynatech) set to a threshold of 1.5, calibration of 1.0 and the 0.5 percent BSA in PBS (Blank) well set to 0.000. The A_{450} data was tabulated in RS-1 on the VAX system, and the standard curve data fitted to a four-parameter logistic model. The unknown samples' concentration were calculated based on the A_{450} data.

E.3 Reconstitution of Recombinant Antibody and Assay

Frozen cells prepared as described in paragraph E.1.10 were thawed in cold lysis buffer [10 mM Tris HCl, pH 7.5, 1 mM EDTA, 0.1M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and lysed by sonication. The lysate was partially clarified by centrifugation for 20 mins at 30,000 rpm. The supernatant was protected from proteolytic enzymes by an additional 1 mM PMSF, and used immediately or stored frozen at –80° C.; frozen lysates were never thawed more than once.

The S-sulfonate of *E. coli* produced anti-CEA heavy chain (γ) was prepared as follows: Recombinant *E. coli* cells transformed with pγCEAtrp207-1* which contained heavy chain as insoluble bodies, were lysed and centrifuged as above; the pellet was resuspended in the same buffer, sonicated and re-centrifuged. This pellet was washed once with buffer, then suspended in 6M guanidine HCl, 0.1M Tris HCl, pH 8, 1 mM EDTA, 20 mg/ml sodium sulfite and 10 mg/ml sodium tetrathionate and allowed to react at 25° for about 16 hrs. The reaction mixture was dialyzed against 8M urea, 0.1M Tris HCl, pH 8, and stored at 4°, to give a 3 mg/ml solution of γ-SSO_3.

650 μl of cell lysate from cells of various *E. coli* strains producing various IgG chains, was added to 500 mg urea. To this was added β-mercaptoethanol to 20 mM, Tris-HCl, pH 8.5 to 50 mM and EDTA to 1 mM, and in some experiments, γ-SSO_3 was added to 0.1 mg/ml. After standing at 25° for 30–90 mins., the reaction mixtures were dialyzed at 4° against a buffer composed of 0.1M sodium glycinate, pH 10.8, 0.5M urea, 10 mM glycine ethyl ester, 5 mM reduced glutathione, 0.1 mM oxidized glutathione. This buffer was prepared from N_2-saturated water and the dialysis was performed in a capped Wheaton bottle. After 16–48 hours, dialysis bags were transferred to 4° phosphate buffered saline containing 1 mM PMSF and dialysis continued another 16–24 hrs. Dialysates were assayed by ELISA as described in paragraph E.2 for ability to bind CEA. The results below show the values obtained by comparison with the standard curve in x ng/ml anti-CEA. Also shown are the reconstitution efficiencies calculated from the ELISA responses, minus the background (108 ng/ml) of cells producing K chain only, and from estimates of the levels of γ and K chains in the reaction mixtures.

	ng/ml anti-CEA	Percent recombination
E. coli W3110 producing IFN-αA (control)	0	—
E. coli (W3110/pKCEAtrp207-1*)	108	—
E. coli (W3110/pKCEAtrp207-1*), plus γ-SSO_3	848	0.33
E. coli (W3110/pKCEAtrp207-1*Δ, pγCEAInt2)	1580	0.76
Hybridoma anti-CEA K-SSO_3 and γ-SSO_3	540	0.40

E.4 Preparation of Chimeric Antibody

FIGS. **11** and **12** show the construction of an expression vector for a chimeric heavy (gamma) chain which comprises the murine anti CEA variable region and human γ-2 constant region.

A DNA sequence encoding the human gamma-2 heavy chain is prepared as follows: the cDNA library obtained by standard techniques from a human multiple myeloma cell line is probed with 5' GGGCACTCGACACAA 3' to obtain the plasmid containing the cDNA insert for human gamma-2 chain (Takahashi, et al., *Cell*, 29: 671 (1982), incorporated herein by reference), and analyzed to verify its identity with the known sequence in human gamma-2 (Ellison, J., et al., *Proc. Natl. Acad. Sci. (USA)*, 79: 1984 (1982) incorporated herein by reference).

As shown in FIG. **11**, two fragments are obtained from this cloned human gamma 2 plasmid (pγ2). The first fragment is formed by digestion with PvuII followed by digestion with Ava III, and purification of the smaller DNA fragment, which contains a portion of the constant region, using 6 percent PAGE. The second fragment is obtained by digesting the pγ2 with any restriction enzyme which cleaves in the 3' untranslated region of γ2, as deduced from the nucleotide sequence, filling in the Klenow and dNTPs, cleaving with Ava III, and isolating the smaller fragment using 6 percent PAGE. (The choice of a two step, two fragment composition to supply the PvuII-3' untranslated fragment provides a cleaner path to product due to the proximity of the AvaIII site to the 3 terminal end thus avoiding additional restriction sites in the gene sequence matching the 3' untranslated region site.) pγCEA207-1* is digested with EcoR 1, treated with Klenow and dNTPs to fill in the cohesive end, and digested with Pvu II, the large vector fragment containing promoter isolated by 6 percent PAGE.

The location and DNA sequence surrounding the PvuII site in the mouse gamma-1 gene are identical to the location and DNA sequence surrounding the PvuII site in the human gamma-2 gene.

The plasmid resulting from a three way ligation of the foregoing fragments, pChim1, contains, under the influence of trp promoter, the variable and part of the constant region of murine anti-CEA gamma 1 chain, and a portion of the gamma 2 human chain. pChim1 will, in fact, express a chimeric heavy chain when transformed into *E. coli*, but one wherein the change from mouse to human does not take place at the variable to constant junction.

FIG. **12** shows modification of pChim1 to construct pChim2 so that the resulting protein from expression will contain variable region from murine anti CEA antibody and constant region from the human γ-2 chain. First, a fragment is prepared from pChim1 by treating with Nco I, blunt ending with Klenow and dNTPs, cleaving with Pvu II, and isolating the large vector fragment which is almost the complete plasmid except for short segment in the constant coding region for mouse anti CEA. A second fragment is prepared from the previously described pγ2 by treating with Pvu II, followed by treating with any restriction enzyme which cleaves in the variable region, blunt ending with Klenow and dNTPs and isolating the short fragment which comprises the junction between variable and constant regions of this chain.

Ligation of the foregoing two fragments produces an intermediate plasmid which is correct except for an extraneous DNA fragment which contains a small portion of the constant region of the murine anti CEA antigen, and a small portion of the variable region of the human gamma chain. This repair can be made by excising the Xba I to Pvu II fragment and cloning into M13 phage as described by Messing et al., *Nucleic Acids Res.* 9: 309 (1981), followed by in vitro site directed deletion mutagenesis as described by Adelman, et al., *DNA*, in press (1983) which is incorporated

herein by reference. The Xba I-Pvu II fragment thus modified is ligated back into the intermediate plasmid to form pChim2. This plasmid then is capable of expressing in a suitable host a cleanly constructed murine variable/human constant chimeric heavy chain.

In an analogous fashion, but using mRNA templates for cDNA construction for human kappa rather than γ chain, the expression plasmid for chimeric light chain is prepared.

The foregoing two plasmids are then double transformed into *E. coli* W3110, the cells grown and the chains reconstituted as set forth in paragraph E.1–E.3 supra.

E.5 Preparation of Altered Murine Anti-CEA Antibody

E.5.1 Construction of Plasmid Vectors for Direct Expression of Altered Murine Anti-CEA Heavy Chain Gene

The cysteine residues, and the resultant disulfide bonds in the region of amino acids 216–230 in the constant region of murine anti-CEA heavy chain are suspected to be important for complement fixation (Klein, et al., *Proc. Natl. Acad. Sci., (USA)*, 78: 524 (1981)) but not for the antigen binding property of the resulting antibody. To decrease the probability of incorrect disulfide bond formation during reconstitution according to the process of the invention herein, the nucleotides encoding the amino acid residues 226–232 which includes codons for three cysteines, are deleted as follows:

A "deleter" deoxyoligonucelotide, 5' CTAACACCATGTCAGGGT is used to delete the relevant portions of the gene from pγCEAtrp207-1* by the procedure of Wallace, et al., *Science*, 209: 1396 (1980) or of Adelman, et al., *DNA* 2, 183 (1983). Briefly, the "deleter" deoxyoligonucelotide is annealed with denatured pγCEAtrp207-1* DNA, and primer repair synthesis carried out in vitro, followed by screening by hybridization of presumptive deletion clones with P^{32} labelled deleter sequence.

E.5.2 Production of Cysteine Deficient Altered Antibody

The plasmid prepared in E.5.1 is transformed into an *E. coli* strain previously transformed with pKCEAtrp207-1* as described above. The cells are grown, extracted for recombinant antibody chains, and the altered antibody reconstituted as described in E.1.10.

E.6 Preparation of Fab

E.6.1 Construction of a Plasmid Vector for Direct Expression of Murine Anti-CEA Gamma 1 Fab Fragment Gene pγCEAFabtrp207-1*

FIG. **13** presents the construction of pγCEAFabtrp207-1*. 5 μg of pBR322 was digested with Hind III, the cohesive ends made flush by treating with Klenow and dNTPs; digested with Pst I, and treated with BAP. The large vector fragment, fragment **I**, was recovered using 6 percent PAGE followed by electroelution.

5 μg of pγCEAtrp207-1* was digested with both BamH I and Pst I and the ~1570 bp DNA fragment (fragment II) containing the trp promoter and the gene sequence encoding the variable region continuing into constant region and further into the anti-CEA gamma 1 chain hinge region, was isolated and purified after electrophoresis.

Expression of the anti-CEA gamma 1 chain Fab fragment rather than complete heavy chain requires that a termination codon be constructed at the appropriate location in the gene. For this, the 260 bp Nco I-Nde I DNA fragment from 20 μg of the pγ298 was isolated and purified. A 13 nucleotide DNA primer, the complement of which encodes the last 3 C-terminal amino acids of the Fab gene and 2 bases of the 3 needed for the stop codon, was synthesized by the phosphotriester method (supra). The probe hybridizes to nucleotides 754 to 767 (FIG. **4**) which has the following sequence:

```
AspCysGlyStop
5' GGGATTGTGGTTG  3'
```

The third base of the stop codon is provided by the terminal nucleotide of the filled-in Hind III site from pBR322 cleavage described above. 500 ng of this primer was used in a primer repair reaction by phosphorylation at the 5' end in a reaction with 10 units T4 DNA kinase containing 0.5 mM ATP in 20 μl, and mixing with ~200 ng of the Nco I-Nde I DNA fragment. The mixture was heat denatured for 3 minutes at 95° and quenched in dry-ice ethanol. The denatured DNA solution was made 60 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C., this primer repair reaction was phenol/$CHCl_3$ extracted, ethanol precipitated, digested with BamH I and the reaction electrophoresed through a 6 percent polyacrylamide gel. ~50 ng of the 181 bp blunt end to BamH I DNA fragment, fragment III, was isolated and purified.

~100 ng of fragment I, ~100 ng each of fragments II and III were ligated overnight and transformed into *E. coli* K12 strain 294. Plasmid DNA from several tetracycline resistant transformants was analyzed for the proper construction and the nucleotide sequence through the repair blunt end filled-in Hind III junction was determined for verification of the TGA stop codon.

E.6.2 Production of Fab Protein

The plasmid prepared in E.6.1 is transformed into an *E. coli* strain previously transformed with pKCEAtrp207-1* as described above. The cells are grown, extracted for recombinant antibody chains and the Fab protein reconstituted as described in E.1.10.

What is claimed is:

1. A process for producing an immunoglobulin molecule or an immunologically functional immunoglobulin fragment comprising at least the variable domains of the immunoglobulin heavy and light chains, in a single host cell, comprising the steps of:

(i) transforming said single host cell with a first DNA sequence encoding at least the variable domain of the immunoglobulin heavy chain and a second DNA sequence encoding at least the variable domain of the immunoglobulin light chain, and

(ii) independently expressing said first DNA sequence and said second DNA sequence so that said immunoglobulin heavy and light chains are produced as separate molecules in said transformed single host cell.

2. The process according to claim **1** wherein said first and second DNA sequences are present in different vectors.

3. The process according to claim **1** wherein said first and second DNA sequences are present in a single vector.

4. A process according to claim **3** wherein the vector is a plasmid.

5. The process according to claim **4** wherein the plasmid is pBR322.

6. The process according to claim **1** wherein the host cell is a bacterium or yeast.

7. The process according to claim **6** wherein the host cell is *E. coli* or *S. cerevisiae*.

8. A process according to claim **7** wherein the host cell is *E. coli* strain X1776 (ATCC No. 31537).

9. A process according to claim **1** wherein the immunoglobulin heavy and light chains are expressed in the host cell and secreted therefrom as an immunologically functional immunoglobulin molecule or immunoglobulin fragment.

10. A process according to claim 1 wherein the immunoglobulin heavy and light chains are produced in insoluble form and are solubilized and allowed to refold in solution to form an immunologically functional immunoglobulin molecule or immunoglobulin fragment.

11. A process according to claim 1 wherein the DNA sequences code for the complete immunoglobulin heavy and light chains.

12. The process according to claim 1 wherein said first or said second DNA sequence further encodes at least one constant domain, wherein the constant domain is derived from the same source as the variable domain to which it is attached.

13. The process according to claim 1 wherein said first or said second DNA sequence further encodes at least one constant domain, wherein the constant domain is derived from a species or class different from that from which the variable domain to which it is attached is derived.

14. The process according to claim 1 wherein said first and second DNA sequences are derived from one or more monoclonal antibody producing hybridomas.

15. A vector comprising a first DNA sequence encoding at least a variable domain of an immunoglobulin heavy chain and a second DNA sequence encoding at least a variable domain of an immunoglobulin light chain wherein said first DNA sequence and said second DNA sequence are located in said vector at different insertion sites.

16. A vector according to claim 15 which is a plasmid.

17. A host cell transformed with a vector according to claim 15.

18. A transformed host cell comprising at least two vectors, at least one of said vectors comprising a DNA sequence encoding at least a variable domain of an immunoglobulin heavy chain and at least another one of said vectors comprising a DNA sequence encoding at least the variable domain of an immunoglobulin light chain.

19. The process of claim 1 wherein the host cell is a mammalian cell.

20. The transformed host cell of claim 18 wherein the host cell is a mammalian cell.

21. A method comprising

a) preparing a DNA sequence consisting essentially of DNA encoding an immunoglobulin consisting of an immunoglobulin heavy chain and light chain or Fab region, said immunoglobulin having specificity for a particular known antigen;

b) inserting the DNA sequence of step a) into a replicable expression vector operably linked to a suitable promoter;

c) transforming a prokaryotic or eukaryotic microbial host cell culture with the vector of step b);

d) culturing the host cell; and

e) recovering the immunoglobulin from the host cell culture, said immunoglobulin being capable of binding to a known antigen.

22. The method of claim 21 wherein the heavy and light chain are the heavy and light chains of anti-CEA antibody.

23. The method of claim 21 wherein the heavy chain is of the gamma family.

24. The method of claim 21 wherein the light chain is of the kappa family.

25. The method of claim 21 wherein the vector contains DNA encoding both a heavy chain and a light chain.

26. The method of claim 21 wherein the host cell is *E. coli* or yeast.

27. The method of claim 26 wherein the heavy chain and light chains or Fab region are deposited within the cells as insoluble particles.

28. The method of claim 27 wherein the heavy and light chains are recovered from the particles by cell lysis followed by solubilization in denaturant.

29. The method of claim 21 wherein the heavy and light chains are secreted into the medium.

30. The method of claim 21 wherein the host cell is a gram negative bacterium and the heavy and light chains are secreted into the periplasmic space of the host cell bacterium.

31. The method of claim 21 further comprising recovering both heavy and light chain and reconstituting light chain and heavy chain to form an immunoglobulin having specific affinity for a particular known antigen.

32. The insoluble particles of heavy chain and light chains or Fab region produced by the method of claim 27.

33. A process for producing an immunoglobulin molecule or an immunologically functional immunoglobulin fragment comprising at least the variable domains of the immunoglobulin heavy and light chains, in a single host cell, comprising:

independently expressing a first DNA sequence encoding at least the variable domain of the immunoglobulin heavy chain and a second DNA sequence encoding at least the variable domain of the immunoglobulin light chain so that said immunoglobulin heavy and light chains are produced as separate molecules in said single host cell transformed with said first and second DNA sequences.

34. The process of claim 9, further comprising the step of attaching the immunoglobulin molecule or immunoglobulin fragment to a label or drug.

35. The process of claim 10, further comprising the step of attaching the immunoglobulin molecule or immunoglobulin fragment to a label or drug.

36. The process of claim 33, further comprising the step of attaching the immunoglobulin molecule or immunoglobulin fragment to a label or drug.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,331,415 B1
DATED : December 18, 2001
INVENTOR(S) : Shmuel Cabilly et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [73], Assignee, please insert -- City of Hope, Duarte, CA (US) --.

Column 19,
Line 56, please change "BamH 1" to -- BamH I --.

Signed and Sealed this

Twenty-fifth Day of June, 2002

Attest:

Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office